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(54) Title: NOVEL ANTIBODIES FOR TREATMENT AND PREVENTION OF INFECTION IN ANIMALS AND MAN (57) Abstract <p>Altered antibodies in which at least parts of the complementary determining regions (CDRs) in the light and/or heavy variable domains of an acceptor monoclonal antibody have been replaced by analogous parts of CDRs from one or more donor monoclonal antibodies, and in which there has been minimal alteration of the acceptor monoclonal antibody light and/or heavy variable domain framework region in order to retain donor monoclonal antibody binding specificity, wherein such donor antibodies have specificity for microorganisms, in particular specificity for respiratory syncytial virus (RSV); a process for preparing such altered antibodies; a pharmaceutical composition comprising a therapeutic, non-toxic amount of such altered antibodies and a pharmaceutically acceptable carrier or diluent; a method of prophylactically or therapeutically treating a microorganism-induced disease state in a human or animal in need thereof which comprises administering an effective amount of such altered antibodies to such human or animal; a specific epitope of the F protein of RSV; monoclonal antibodies directed against such epitope; Fab fragments of such monoclonal antibodies; a pharmaceutical composition comprising a therapeutic, non-toxic amount of such monoclonal antibodies or Fab fragments and a pharmaceutically acceptable carrier or diluent; and a method of prophylactically or therapeutically treating RSV infection in a human or animal in need thereof which comprises administering an effective amount of such monoclonal antibodies or Fab fragments to such human or animal.</p>			

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- 1 -

NOVEL ANTIBODIES FOR TREATMENT AND PREVENTION OF INFECTION IN ANIMALS AND MAN

5

BACKGROUND OF THE INVENTION

There has long been a need for effective agents for prevention and treatment of infection in animals and man. Typical methods comprise administration of chemical agents which inhibit the growth of microorganisms allowing the immune system to eradicate the infectious agent. Whilst natural and synthetic chemicals have been particularly effective as treatments for bacterial infection, the emergence of resistant strains has proved frequent and problematic. For viral infections, chemical agents have had limited effect and the severity of disease is usually correlated with immune system status.

For many years, the effectiveness of serum from immune individuals on prevention and treatment of infectious disease has been known. However, it is well known that the antibodies within human immune sera which are responsible for effective treatment, i.e., the neutralising antibody component, are only a very small fraction of the total sera antibody. Furthermore, the use of immune sera has been limited by low neutralising antibody levels, by the scarcity of immune donors, by the cost of treatment and more recently by the risk of adventitious spread of disease through microorganisms in donor sera.

The development of monoclonal antibody technology provided the means for development and production of pure murine monoclonal antibodies in large quantities from cell lines devoid of pathogenic microorganisms. With this technique it was possible to provide monoclonal antibodies which interacted with pathogenic organisms, some of which monoclonal antibodies could prevent the growth of the target microorganisms in infected mice. Unfortunately, it is not possible to predict from *in vitro* studies which antibodies will be most effective at *in vivo* killing of microorganisms. Many

- 2 -

monoclonal antibodies with high binding affinity for their target in an in vitro setting are not effective in vivo. In fact, in some cases where antibodies are effective at preventing growth of the microorganisms under laboratory conditions, they prove ineffective in the in vivo environment.

5

The impact and limitations of murine monoclonal antibodies for treatment of infectious disease is illustrated by the case of respiratory syncytial virus (RSV) infection. RSV is the major cause of lower respiratory tract infection in infants in the first year of life and a significant cause of respiratory disease in young cattle. In man, most attempts to vaccinate against RSV infection have failed, and treatment of RSV infection with chemical drugs such as ribavirin is only partially effective. Murine monoclonal antibodies specific for RSV have been shown to be effective in prevention and treatment of RSV in mice. However, the use of murine monoclonal antibodies for treatment and prevention of RSV in non-murine species is potentially limited by the immune response of these species to the "foreign" murine antibody, i.e., immune responses in humans against murine antibodies have been shown to both immunoglobulin constant and variable regions (human anti-mouse antibodies). Therefore, non-immunogenic variants of monoclonal antibodies where the immunoglobulin constant and variable regions contain amino acid sequences recognised as "self" by the RSV infected recipient are needed for effective prevention and treatment of RSV infection.

25 Recombinant DNA technology has provided the ability to alter antibodies in order to substitute specific immunoglobulin (Ig) regions from one species with regions from another. Patent Cooperation Treaty Patent Application No. PCT/GB85/00392 (Neuberger et al and Celltech Limited) describes a process whereby the complementary heavy and light chain variable domain of an Ig molecule from one species may be combined with the complementary heavy and light chain Ig constant domains from another species. This process may be used, for example, to alter murine monoclonal antibodies directed against a specific human disease. Such alteration is effected by substitution of the murine antibody constant region domains with human IgG constant region domains to create a "chimeric" antibody to

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- 3 -

be potentially used for treatment of such human disease. However, such chimeric antibodies will still potentially elicit an immune response in humans against the murine (i.e, "foreign") variable regions.

- 5 British Patent Application Publication Number GB2188638A (Winter) describes a process whereby antibodies are altered by substitution of their complementarity determining regions (CDRs) from one species with those from another. This process may be used, for example, to substitute the CDRs from human heavy and light chain Ig variable region domains with
10 alternative CDRs from murine variable region domains. These altered Ig variable regions may subsequently be combined with human Ig constant regions to create antibodies which are totally human in composition except for the substituted murine CDRs. Such murine CDR-substituted antibodies are likely to elicit a considerably reduced immune response in
15 humans compared to chimeric antibodies because they contain considerably less murine components. However, as stated in British Patent Application Publication Number GB2188638A, merely replacing one or more CDRs with complementary CDRs from another species which are specific for a desired disease may not always result in an altered antibody which retains the
20 antigen binding capacity of complementary CDRs. The British Patent Application proposes that by "routine experimentation or by trial and error", a functional altered antibody with antigen binding capacity may be obtained. However, no description of the nature of the routine experimentation or the trial and error process needed to obtain the desired
25 antibody is provided, and there is a suggestion that successive replacements of CDRs from different sources should be attempted.

- 30 Examination of the three-dimensional structures of several IgGs has led to the conclusion that the Ig variable regions of heavy and light chains each comprise three looped structures (which include the CDRs) supported on a sheet-like structure termed the variable region framework. The predominant definition of what comprises a CDR and what comprises a framework is based upon amino acid sequences of a number of Igs.

- 4 -

In three dimensional configuration, the aforementioned loop structures and CDRs between a mouse and human antibody do not correspond exactly although there is considerable overlap. Therefore it appears that, in some cases, the transfer of antigen binding specificity by replacement of CDRs
5 may require the additional replacement of residues adjacent to the defined CDRs. For example, it has been hypothesized that , in certain cases, variable region framework amino acid residues may be important in antigen binding through direct interaction with CDRs (See, Amit et al., Science, 233 (1986) pp 747-753; Queen et al., Proc. Natl. Acad. Sci., 86 (1989) pp10029-
10 10033; and Protein Design Labs, Patent Cooperation Treaty Patent Application Publication Number WO9007861, published July 26, 1990). In the Queen et al. reference, the authors selected human variable regions for murine CDR-replacement on the basis of maximum homology to the murine variable region comprising the CDRs used for the replacement. In addition,
15 on the basis of computer modelling, the Queen et al. authors utilized a human framework for CDR replacement which included several murine framework amino acids thought to interact with the murine CDRs. The resultant altered antibody, whilst retaining antigen binding capacity, contained additional murine framework amino acids. Such additional
20 murine framework amino acids might contribute to an enhanced immune response to the altered antibody in humans.

In addition, previous studies (see, e.g., Riechmann,et al., Nature, 332 (1988), p323-327) have demonstrated that the use of reshaping can be used
25 to transfer in vitro high affinity binding from mouse to human antibodies, but it has not previously been shown that it is possible to provide the combination of properties required for preservation of effective prevention of growth of human respiratory syncytial virus (RSV) in vivo.

30 Therefore, there is a need for altered antibodies with minimal immunogenicity for the prevention and treatment of infectious disease. In addition, there is a need for a defined process to produce such altered antibodies without radical alteration of variable region frameworks and the associated effect on immunogenicity. The present invention provides
35 altered antibodies for prevention and treatment of infectious disease and a

- 5 -

process for their production by introducing only critical variable region framework modifications.

RSV, which is in the genus *Pneumovirus* of the *Paramyxoviridae* family, is a major cause of lower respiratory tract infections in young children. Primary infection gives an incomplete immunity, and reinfection is frequently observed during childhood. The role of immune mechanisms in the human disease have not been clarified. Previous attempts to develop effective vaccines with attenuated or killed RSV have met with failure, i.e., not only were the children unprotected, but subsequent infections with RSV sometimes resulted in more severe diseases than in non-immunized controls. RSV infection is also a major cause of respiratory infection in young cattle.

Recently, certain immunological and molecular information has been obtained regarding the antigenic and functional properties of RSV proteins. The RSV fusion protein (F) and the RSV attachment protein (G) have been identified as the major viral antigens, and their genes have been cloned and sequenced. Two antigenically distinct subgroups of human RSV, designated A and B, have been described. The antigenic differences between A and B subgroups reside mainly on the RSV G protein. In contrast, the RSV F protein has a high degree of genetic and antigenic homology between the two subgroups, and various strains within these subgroups.

Monoclonal antibodies (mAbs) directed against both envelope glycoproteins (F and G) of RSV have been demonstrated to neutralize the virus. (See, Walsh & Hruska, *J. Virology*, **47**, 171-177 (1983); and Walsh et al., *J. Gen. Virology*, **65**, 761-767 (1984)). However, *in vitro* and *in vivo* studies with mAbs or with vaccinia virus recombinants expressing F protein indicated that this protein is the most important antigen in inducing cross-protective immunity. (See, Johnson et al., *J. Virology*, **61**, 3163-3166 (1987); Olmsted et al., *Proc. Nat. Acad. Sci., USA*, 7462-7466 (1986); Wertz

- 6 -

et al., J. Virology, 61, 293-310 (1987); and Walsh et al., Infection and Immunity, 43, 756-758 (1984)). Several authors have identified different antigenic sites in the F protein and have shown that at least three of these antigenic sites are involved in neutralization.

5 Two or three neutralizing epitopes have been located on the F protein in different ways. Using escape mutant viruses, Lopez et al., J. Virology, 64, 927-930 (1990) have shown that two amino acid residues (i.e., 262-Asn and 268-Asn) of the F₁ subunit of the F protein are essential for the integrity of a particular neutralizing

10 epitope. Another highly conserved neutralizing epitope has been mapped with synthetic peptides to residues 221-Ile to 232-Glu of the F₁ subunit of the F protein by Trudel et al., J. General Virology, 68, 2273-2280 (1987). Finally, a recent analysis by the Pepscan procedure identified an epitope at positions 483-Phe to 488-Phe of

15 the F₁ subunit of the F protein, which epitope could correspond to another neutralizing epitope. (See, Scopes et al., J. General Virology, 71, 53-59 (1990)).

There is a need for the development of new therapies for the treatment and

20 prevention of RSV infection. A neutralizing and protective epitope of an RSV viral antigen could prove useful in the generation of monoclonal antibodies useful for the prophylaxis and/or treatment of RSV infection. The present invention provides such a novel epitope on the RSV F protein which is recognised by a neutralizing and protective antibody *in vivo*.

25

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the DNA sequence and corresponding amino acid sequence of the RSV19 heavy chain variable region (VH). The CDR sequences are

30 boxed. The first eight and last eleven amino acids, as underlined, correspond to sequences of the oligonucleotide primers used.

Figure 2 shows the DNA sequence and corresponding amino acid sequence of the RSV19 light chain variable region (VK). The CDR sequences are

- 7 -

boxed. The first eight and last six amino acids, as underlined, correspond to sequences of the oligonucleotide primers used.

5 Figure 3 shows the basic plasmid pHuRSV19VH comprising a human Ig heavy chain variable region framework and CDRs derived from mouse RSV19.

10 Figure 4 shows the basic plasmid pHuRSV19VK comprising a human Ig light chain variable region framework and CDRs derived from mouse RSV19.

15 Figure 5 shows the derived Ig variable region amino acid sequences encoded by RSV19VH, RSV19VK, pHuRSVVH and pHuRSV19VK, and derivations of pHuRSV19VH.

Figure 6 shows an ELISA analysis of the binding of HuRSV19VH/VK antibody and its derivative, HuRSV19VHFNS/VK, to RSV antigen.

20 Figure 6A shows that there is little if any difference between the ability of the RSV19 and HuRSV19VHFNS/HuRSV19VK antibodies to bind to intact, non-denatured RS virus.

25 Figure 7 shows that mAb RSV19 binds to two synthetic peptides consisting of, respectively, amino acid residues 417-432 and 422-438 of the F protein.

SUMMARY OF THE INVENTION

30 The present invention relates to altered antibodies in which at least parts of the complementarity determining regions (CDRs) in the light and/or heavy variable domains of an acceptor monoclonal antibody have been replaced by analagous parts of CDRs from one or more donor monoclonal antibodies, and in which there may or may not have been minimal alteration of the acceptor monoclonal antibody light and/or heavy variable domain
35 framework region in order to retain donor monoclonal antibody binding

- 8 -

specificity, wherein such donor antibodies have specificity for microorganisms, in particular specificity for respiratory syncytial virus (RSV). The present invention also relates to a process for preparing such altered antibodies; a pharmaceutical composition comprising a therapeutic, non-toxic amount of such altered antibodies and a pharmaceutically acceptable carrier or diluent; and a method of prophylactically or therapeutically treating a microorganism-induced disease state in a human or animal in need thereof which comprises administering an effective amount of such altered antibodies to such human or animal. Preferably the altered antibodies of the invention will be produced by recombinant DNA technology. The altered antibody of the present invention may comprise a complete antibody molecule (having full length heavy and light chains) or any fragment thereof, such as the Fab or (Fab')₂ fragment, a light chain or heavy chain dimer, or any minimal recombinant fragment thereof such as an Fv or a SCA (single-chain antibody) or any other molecule with the same specificity as the altered antibody of the invention. Alternatively, the altered antibody of the invention may have attached to it an effector or reporter molecule. For instance, the altered antibody of the invention may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively the procedure of recombinant DNA technology may be used to produce an altered antibody of the invention in which the Fc fragment or CH3 domain of a complete antibody molecule has been replaced by an enzyme or toxin molecule. The remainder of the altered antibody may be derived from any suitable human immunoglobulin. However, it need not comprise only protein sequences from the human immunoglobulin. For instance a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a polypeptide effector or reporter molecule.

Another aspect of this invention is the discovery of a specific epitope of the F (fusion) protein of RSV which has been demonstrated to be a target for monoclonal antibodies which both protect and cure mice of infection by RSV. In addition, it has also been demonstrated that Fab fragments of such monoclonal antibodies protect mice from in vivo infection. Thus, the present

- 9 -

invention also relates to such specific epitope of the F protein of RSV; monoclonal antibodies directed against such epitope; and Fab fragments of such monoclonal antibodies. In addition, this invention relates to a pharmaceutical composition comprising a therapeutic, non-toxic amount of such monoclonal antibodies or Fab fragments and a pharmaceutically acceptable carrier or diluent; and a method of prophylactically or therapeutically treating RSV infection in a human or animal in need thereof which comprises administering an effective amount of such monoclonal antibodies or Fab fragments to such human or animal.

The present invention provides altered antibodies with specificity for microorganisms, and the DNA coding for such antibodies. These antibodies comprise Ig constant regions and variable regions from one source, and one or more CDRs from a different source.

In addition, amino acid substitutions in the variable region frameworks are described which are critical for antigen binding affinity. The invention also provides vectors producing the altered antibodies in mammalian cell hosts.

The present invention particularly applies to the provision of altered antibodies with the combination of properties required for the prevention and treatment of infections in animals and man. For example, non-human antibodies with specificity for micro organisms may be altered to produce "humanised" antibodies which elicit a minimal immune response in humans. In particular, the invention provides "humanised" antibodies with specificity for RSV which are shown to be effective in an animal model for RSV infection in humans and to recognise a large variety of human clinical isolates of RSV.

The present invention also provides a method for effecting minimal modifications to the amino acids of variable region frameworks in order to retain the antigen binding capacity of CDRs from a different source. The method involves stepwise alteration and testing of individual amino acids in the variable region framework potentially critical for antigen binding

- 10 -

affinity. The method avoids major introduction of framework amino acids from the same source as CDRs.

DETAILED DESCRIPTION OF THE INVENTION

5 As used herein, the term "humanized antibody" refers to a molecule having its complementarity determining regions (and, perhaps, minimal portions of its light and/or heavy variable domain framework region) derived from an immunoglobulin from a non-human species, the remaining immunoglobulin-
10 derived parts of the molecule being derived from a human immunoglobulin.

The present invention relates to altered antibodies in which at least parts of the complementarity determining regions (CDRs) in the light and/or heavy variable domains of an acceptor monoclonal antibody have been replaced by
15 analagous parts of CDRs from one or more donor monoclonal antibodies, and in which there may or may not have been minimal alteration of the acceptor monoclonal antibody light and/or heavy variable domain framework region in order to retain donor monoclonal antibody binding specificity, wherein such donor antibodies have specificity for
20 microorganisms, in particular specificity for respiratory syncytial virus (RSV). The present invention also relates to a process for preparing such altered antibodies; a pharmaceutical composition comprising a therapeutic, non-toxic amount of such altered antibodies and a pharmaceutically acceptable carrier or diluent; and a method of prophylactically or
25 therapeutically treating a microorganism-induced disease state in a human or animal in need thereof which comprises administering an effective amount of such altered antibodies to such human or animal.

The altered antibodies of the invention may be produced by the following
30 process:

(a) producing, by conventional techniques, in an expression vector an operon having a DNA sequence which encodes an antibody heavy or light chain wherein at least the CDRs (and those minimal portions of the acceptor
35 monoclonal antibody light and/or heavy variable domain framework region

- 11 -

required in order to retain donor monoclonal antibody binding specificity) of the variable domain are derived from a non-human immunoglobulin, such as that produced by RSV19, and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin,
5 thereby producing the vector of the invention;

(b) producing, by conventional techniques, in an expression vector an operon having a DNA sequence which encodes a complementary antibody light or heavy chain wherein at least the CDRs (and those minimal portions of the
10 acceptor monoclonal antibody light and/or heavy variable domain framework region required in order to retain donor monoclonal antibody binding specificity) of the variable domain are derived from a non-human immunoglobulin, such as that produced by RSV19, and the remaining immunoglobulin-derived parts of the antibody chain are derived from a
15 human immunoglobulin, thereby producing another vector of the invention;

(c) transfecting a host cell by conventional techniques with the or each vector to create the transfected host cell of the invention;

20 (d) culturing the transfected cell by conventional techniques to produce the altered antibody of the invention.

The host cell may be transfected with two vectors of the invention, the first vector containing an operon encoding a light chain-derived polypeptide and
25 the second vector containing an operon encoding a heavy chain-derived polypeptide. Preferably the vectors are identical except in so far as the coding sequences and selectable markers are concerned so to ensure as far as possible that each polypeptide chain is equally expressed. Alternatively, a single vector of the invention may be used, the vector including the
30 sequence encoding both light chain- and heavy chain-derived polypeptides. The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both.

- 12 -

The host cell used to express the altered antibody of the invention is preferably a eukaryotic cell, most preferably a mammalian cell, such as a CHO cell or a myeloid cell.

5 The general methods by which the vectors of the invention may be constructed, transfection methods required to produce the host cell of the invention, and culture methods necessary to produce the altered antibody of the invention from such host cell are all conventional techniques. Likewise, once produced, the altered antibodies of the invention may be purified
10 according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like.

An example of the altered antibody of the invention are humanised
15 antibodies derived from the murine monoclonal antibody RSV19 such as HuRSV19VH/VK and HuRSV19VHFNS/HuRSV19VK which are described in the Examples. Such antibodies are useful in treating, therapeutically or prophylactically, a human against human RSV infection. Therefore, this invention also relates to a method of treating, therapeutically or
20 prophylactically, human RSV infection in a human in need thereof which comprises administering an effective, human RSV infection treating dose such altered antibodies to such human.

The altered antibodies of this invention may also be used in conjunction
25 with other antibodies, particularly human monoclonal antibodies reactive with other markers (epitopes) responsible for the disease against which the altered antibody of the invention is directed.

The altered antibodies of this invention may also be used as separately
30 administered compositions given in conjunction with chemotherapeutic or immunosuppressive agents. The appropriate combination of agents to utilized can readily be determined by one of skill in the art using conventional techniques. As an example of one such combination, the altered antibody of the invention known as HuRSV19VHFNS/HuRSV19VK

- 13 -

may be given in conjunction with the antiviral agent ribavirin in order to facilitate the treatment of RSV infection in a human.

One pharmaceutical composition of the present invention comprises the use
5 of the antibodies of the subject invention in immunotoxins, i.e., molecules
which are characterized by two components and are particularly useful for
killing selected cells in vitro or in vivo. One component is a cytotoxic agent
which is usually fatal to a cell when attached or absorbed. The second
component, known as the "delivery vehicle" provides a means for delivering
10 the toxic agent to a particular cell type, such as cells comprising a
carcinoma. The two components are commonly chemically bonded together
by any of a variety of well-known chemical procedures. For example, when
the cytotoxic agent is a protein and the second component is an intact
immunoglobulin, the linkage may be by way of heterobifunctional cross-
15 linkers, e.g., carbodiimide, glutaraldehyde, and the like. Production of
various immunotoxins is well-known in the art.

A variety of cytotoxic agents are suitable for use in immunotoxins, and may
include, among others, radionuclides, chemotherapeutic drugs such as
20 methotrexate, and cytotoxic proteins such as ribosomal inhibiting proteins
(e.g., ricin).

The delivery component of the immunotoxin will include the human-like
immunoglobulins of the present invention. Intact immunoglobulins or their
25 binding fragments, such as Fab, are preferably used. Typically, the
antibodies in the immunotoxins will be of the human IgM or IgG isotype,
but other mammalian constant regions may be utilized if desired.

The altered antibodies and pharmaceutical compositions of the invention
30 are particularly useful for parenteral administration, i.e., subcutaneously,
intramuscularly or intravenously. The compositions for parenteral
administration will commonly comprise a solution of the altered antibody of
the invention or a cocktail thereof dissolved in an acceptable carrier,
preferably an aqueous carrier. A variety of aqueous carriers may be
35 employed, e.g., water, buffered water, 0.4% saline, 0.3% glycine, and the

- 14 -

like. These solutions are sterile and generally free of particulate matter. These solutions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions
5 such as pH adjusting and buffering agents, etc. The concentration of the the altered antibody of the invention in such pharmaceutical formulation can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., according to the particular mode of
10 administration selected.

Thus, a pharmaceutical composition of the invention for intramuscular injection could be prepared to contain 1 mL sterile buffered water, and 50 mg of an altered antibody of the invention. Similarly, a pharmaceutical
15 composition of the invention for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of an altered antibody of the invention. Actual methods for preparing parenterally administrable compositions are well known or will be apparent to those skilled in the art and are described in more detail in, for example,
20 Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania.

The altered antibodies of the invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been
25 shown to be effective with conventional immune globulins and art-known lyophilization and reconstitution techniques can be employed.

Depending on the intended result, the pharmaceutical composition of the invention can be administered for prophylactic and/or therapeutic
30 treatments. In therapeutic application, compositions are administered to a patient already suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. In prophylactic applications, compositions containing the present antibodies or a cocktail thereof are administered to a patient not already in a disease state to
35 enhance the patient's resistance.

- 15 -

Single or multiple administrations of the pharmaceutical compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical composition of the invention
5 should provide a quantity of the altered antibodies of the invention sufficient to effectively treat the patient.

It should also be noted that the altered antibodies of this invention may be used for the design and synthesis of either peptide or non-peptide
10 compounds (mimetics) which would be useful in the same therapy as the antibody. See, e.g., Saragovi et al., Science, 253, 792-795 (1991).

Another aspect of this invention is the discovery of a specific epitope of the F (fusion) protein of RSV which has been demonstrated to be a target for
15 monoclonal antibodies which both protect and cure mice of infection by RSV. In addition, it has also been demonstrated that Fab fragments of such monoclonal antibodies protect mice from in vivo infection. Thus, the present invention also relates to such specific epitope of the F protein of RSV;
monoclonal antibodies directed against such epitope; and Fab fragments of
20 such monoclonal antibodies. In addition, this invention relates to a pharmaceutical composition comprising a therapeutic, non-toxic amount of such monoclonal antibodies or Fab fragments and a pharmaceutically acceptable carrier or diluent; and a method of prophylactically or
therapeutically treating RSV infection in a human or animal in need thereof
25 which comprises administering an effective amount of such monoclonal antibodies or Fab fragments to such human or animal.

The present invention provides altered antibodies with specificity for microorganisms, and the DNA coding for such antibodies. These antibodies
30 comprise Ig constant regions and variable regions from one source, and one or more CDRs from a difference source.

In addition, amino acid substitutions in the variable region frameworks are described which are critical for antigen binding affinity. The invention also
35 provides vectors producing the altered antibodies in mammalian cell hosts.

- 16 -

The present invention particularly applies to the provision of altered antibodies with the combination of properties required for the prevention and treatment of infections in animals and man. For example, non-human
5 antibodies with specificity for micro organisms may be altered to produce "humanised" antibodies which elicit a minimal immune response in humans. In particular, the invention provides "humanised" antibodies with specificity for RSV which are shown to be effective in an animal model for RSV infection in humans and to recognise a large variety of human clinical
10 isolates of RSV.

The present invention also provides a method for effecting minimal modifications to the amino acids of variable region frameworks in order to retain the antigen binding capacity of CDRs from a different source. The
15 method involves stepwise alteration and testing of individual amino acids in the variable region framework potentially critical for antigen binding affinity. The method avoids major introduction of framework amino acids from the same source as CDRs.

20 The following examples are offered by way of illustration, not by limitation.

EXAMPLES

In the following examples all necessary restriction enzymes, plasmids, and
25 other reagents and materials were obtained from commercial sources unless otherwise indicated.

In the following examples, unless otherwise indicated, all general cloning, ligation and other recombinant DNA methodology was performed as
30 described in "Molecular Cloning, A Laboratory Manual (1982) eds T. Maniatis et. al., published by Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (hereinafter referred to as "Maniatis et al.").

- 17 -

In the following examples, the following abbreviations may be employed:

	dCTP	deoxycytidine triphosphate
	dATP	deoxyadenosine triphosphate
	dGTP	deoxyguanosine triphosphate
5	dTTP	deoxythysiodine triphosphate
	DTT	dithiothreitol
	C	cytosine
	A	adenine
	T	thymine
10	G	guanine
	DMEM	Dulbecco's modified Eagle's medium
	PBST	Phosphate buffered saline containing 0.02% Tween 20 (pH 7.5)

15 ALTERED ANTIBODIES

Examples 1-3 describe the preparation of the altered antibodies of the invention.

EXAMPLE 1-PRODUCTION OF ALTERED ANTIBODIES SPECIFIC FOR RSV

The source of the donor CDRs utilized to prepare these altered antibodies
20 was a murine monoclonal antibody, RSV19, specific for the fusion (F)
protein of RSV. The RSV19 hybridoma cell line was obtained from Dr.
Geraldine Taylor, Institute for Animal Health, Compton Laboratory,
Compton, Near Newbury, Berks, RG16 0NN, England. Methodology for the
isolation of hybridoma cell lines secreting monoclonal antibodies specific for
25 RSV is described by Taylor et al., Immunology, 52 (1984) p137-142.

Cytoplasmic RNA was prepared by the method of Favaloro et. al., (1980)

Methods in Enzymology, Vol. 65, p.718-749, from the RSV19 hybridoma cell
line, and cDNA was synthesized using Ig variable region primers as follows:

30 for the Ig heavy chain variable (VH) region, the primer
VH1FOR (5'TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG3')
was used, and
for the Ig light chain variable region (VK), the primer
VK1FOR (5'GTTAGATCTCCAGCTTGGTCCC3')
35 was used.

- 18 -

cDNA synthesis reactions consisted of 20mg RNA, 0.4mM VH1FOR or VK1FOR, 250mM each of dATP, dCTP, dGTP and dTTP, 50mM Tris-HCl pH 7.5, 75mM KCl, 10mM DTT, 3mM MgCl₂ and 27 units RNase inhibitor (Pharmacia, Milton Keynes, United Kingdom) in a total volume of 50ml. Samples were heated at 70°C for 10 minutes (min) and slowly cooled to 42°C over a period of 30 min. Then, 100m MMLV reverse transcriptase (Life Technologies, Paisley, United Kingdom) was added and incubation at 42°C continued for 1 hour.

10

VH and VK cDNAs were then amplified using the polymerase chain reaction (PCR) as described by Saiki, et al., Science, 239 (1988), p487-491. For such PCR, the primers used were:

VH1FOR;
VK1FOR;
VH1BACK (5'AGGTSMARCTGCAGSAGTCWGG3'); and
VK1BACK (5'GACATTCAGCTGACCCAGTCTCCA3'),

where M = C or A, S = C or G, and W = A or T.

Primers VH1FOR, VK1FOR, VH1BACK and VK1BACK, and their use for PCR-amplification of mouse Ig DNA, is described by Orlandi et al., Proc. Nat. Acad. Sci. USA, 86, 3833-3937 (1989).

For PCR amplification of VH, DNA/primer mixtures consisted of 5ml RNA/cDNA hybrid, and 0.5mM VH1FOR and VH1BACK primers. For PCR amplifications of VK, DNA/primer mixtures consisted of 5ml RNA/cDNA hybrid, and 0.5mM VH1FOR and VK1BACK primers. To these mixtures was added 200 mM each of dATP, dCTP, dGTP and dTTP, 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM MgCl₂, 0.01% (w/v) gelatin, 0.01% (v/v) Tween 20, 0.01% (v/v) Nonidet P40 and 2 units Taq DNA polymerase (United States Biochemicals-Cleveland, Ohio, USA). Samples were subjected to 25 thermal cycles of PCR at 94°C, 1 min; 60°C, 1 min; 72°C, 2 min; ending with 5 min at 72°C. For cloning and sequencing, amplified VH DNA was purified on a low melting point agarose gel and by Elutip-d column chromatography (Schleicher and Schuell-Dussel, Germany) and cloned into phage M13

- 19 -

(Pharmacia-Milton Keynes, United Kingdom). The general cloning and ligation methodology was as described in "Molecular Cloning, A Laboratory Manual (1982) eds T. Maniatis et. al., published by Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (hereinafter referred to as "Maniatis et al.". VH DNA was either directly ligated into the SmaI site of M13 mp18/19 (Pharmacia-Milton Keynes, UK) or, following digestion with PstI, into the PstI site of M13tg131 (Amersham International-Little Chalfont, UK). Amplified VK was similarly gel purified and cloned by the following alternatives:

- 10 PvuII digest into M13mp19 (SmaI site)
- PvuII and BglII digest into M13mp18/19 (SmaI - BamHI site)
- PvuII and BglII digest into M13tg131 (EcoRV - BglII site)
- BglII digest into M13tg131 (SmaI - BglII site)

- 15 The resultant collections of overlapping clones were sequenced by the dideoxy method (Sanger, et al., Proc. Nat. Acad. Sci. USA, 74 (1977) p5463-5467) using Sequenase (United States Biochemicals-Cleveland, Ohio, USA).

From the sequence of RSV19 VH and VK domains, as shown in Figure 1 and 20 2 respectively, the CDR sequences were elucidated in accordance with the methodology of Kabat et al., in Sequences of Proteins of Immunological Interest (US Dept of Health and Human Services, US Government Printing Office, (1987)) utilizing computer assisted alignment with other VH and VK sequences.

25 Transfer of the murine RSV19 CDRs to human frameworks was achieved by site directed mutagenesis. The primers used were:

- VHCDR1 5'CTGTCTCACCCAGTGCATATAGTAGTCGCTGAAGGTGAA
GCCAGACACGGT3'
- 30 VHCDR2 5'CATTGTCACTCTGCCCTGGAACCTCGGGGCATATGGAA
CATCATCATTCTCAGGATCAATCCA3'

- 20 -

VHCDR3 5' CCCTTGGCCCCAGTGGTCAAAGTCACTCCCCCATCTT
GCACAATA3'

VKCDR1 5' CTGCTGGTACCATTCTAAATAGGTGTTTCCATCAGTATGT
ACAAGGGTCTGACTAGATCTACAGGTGATGGTCA3'

5 VKCDR2 5' GCTTGGCACACCAGAAAATCGGTTGGAAACTCTGTAG
ATCAGCAG3'

VKCDR3 5' CCCTTGGCCGAACGTCCGAGGAAGATGTGAACCTTGAA
AGCAGTAGTAGGT3'

- 10 The DNA templates for mutagenesis comprised human framework regions derived from the crystallographically solved proteins, NEW (described by Saul, et al., J. Biol. Chem. **53** (1978), p585-597) with a substitution of amino acid 27 from serine to phenylalanine (See, Riechmann et al., loc. cit.) and REI (described by Epp et al, Eur J. Biochem. **45** (1974), p513-524) for
15 VH and VK domains, respectively. M13 based templates comprising human frameworks with irrelevant CDRs were prepared as described by Riechmann et al., Nature, **332** (1988).

- Oligonucleotide site directed mutagenesis of the human VH and VK genes
20 was based on the method of Nakamaye et al., Nucl. Acids Res. **14** (1986) p9679-9698.

- To 5mg of VH or VK single-stranded DNA in M13 was added a two-fold molar excess of each of the three VH or VK phosphorylated oligonucleotides
25 encoding the three mouse CDR (complementarity determining region) sequences. Primers were annealed to the template by heating to 70°C and slowly cooled to 37°C. To the annealed DNA was added 6u Klenow fragment (Life Technologies, Paisley, UK); 6u T4 DNA ligase (Life Technologies, Paisley, UK); 0.5mM of each of the following nucleoside
30 triphosphates (dATP, dGTP, dTTP and 2'-deoxycytidine 5'-0-(1-thiotriphosphate) (thiodCTP); 60mM Tris-HCl (pH 8.0); 6mM MgCl₂; 5mM DTT (Sigma, Poole, UK); and 10mM ATP in a reaction volume of 50ml. This mixture was incubated at 16°C for 15 hours (h). The DNA was then ethanol precipitated and digested with 5 units NciI (Life Technologies, Paisley, UK)
35 which nicks the parental strand but leaves the newly synthesised strand

- 21 -

containing thiodCTP intact. The parental strand was then removed by digesting for 30 min with 100 units exonuclease III (Pharmacia, Milton Keynes, United Kingdom) in 50 ml of 60mM Tris-HCl (pH 8.0), 0.66mM MgCl₂, and 1mM DTT. The DNA was then repaired through addition of 3 units of DNA polymerase I (Life Technologies, Paisley, UK), 2 units T4 DNA ligase in 50 ml of 60mM Tris-HCl (pH 8.0), 6mM MgCl₂, 5mM DTT, 10mM ATP and 0.5mM each of dATP, dCTP, dGTP and dTTP. The DNA was transformed into competent *E. coli* TG1 cells (Amersham International, Little Chalfont, UK) by the method of Maniatis et al. Single-stranded DNA was prepared from individual plaques and sequenced by the method of Messing (1983) Methods in Enzymology, 101, p. 20-78. If only single or double mutants were obtained, then these were subjected to further rounds of mutagenesis (utilizing the methodology described above) by using the appropriate oligonucleotides until the triple CDR mutants were obtained.

The CDR replaced VH and VK genes were cloned in expression vectors (by the method of Maniatis et al.) to yield the plasmids shown in Figures 3 and 4 respectively, and such plasmids were termed pHuRSV19VH and pHuRSV19VK. For pHuRSV19VH, the CDR replaced VH gene together with the Ig heavy chain promoter (Figures 3 and 4), appropriate splice sites and signal peptide sequences (Figures 3 and 4) were excised from M13 by digestion with HindIII and BamHI, and cloned into an expression vector containing the murine Ig heavy chain enhancer (Figures 3 and 4), the SV40 promoter (Figures 3 and 4), the gpt gene for selection in mammalian cells (Figures 3 and 4) and genes for replication and selection in *E. coli* (Figures 3 and 4). A human IgG1 constant region was then added as a BamHI fragment (Figures 3 and 4). The construction of the pHuRSV19VK plasmid was essentially the same except that the gpt gene was replaced by the hygromycin resistance gene (Figures 3 and 4) and a human kappa chain constant region was added (Figures 3 and 4).

10mg of pHuRSV19VH and 20mg of pHuRSV19VK were digested with PvuI utilizing conventional techniques. The DNAs were mixed

- 22 -

together, ethanol precipitated and dissolved in 25ml water. Approximately 10^7 YB2/0 cells (from the American Type Culture Collection, Rockville, Maryland, USA) were grown to semi-confluency, harvested by centrifugation and resuspended in 0.5ml DMEM (Gibco, Paisley, UK) together with the digested DNA in a cuvette. After 5 min on ice, the cells were given a single pulse of 170V at 960uF (Gene-Pulser, Bio-Rad-Richmond, California, USA) and left in ice for a further 20 min. The cells were then put into 20 ml DMEM plus 10% foetal calf serum and allowed to recover for 48h. After this time, the cells were distributed into a 24-well plate and selective medium applied (DMEM, 10% foetal calf serum, 0.8mg/ml mycophenolic acid, and 250mg/ml xanthine). After 3-4 days, the medium and dead cells were removed and replaced with fresh selective medium. Transfected clones were visible with the naked eye 10-12 days later.

The presence of human antibody in the medium of wells containing transfected clones was measured by conventional ELISA techniques. Micro-titre plates were coated overnight at 4°C with goat anti-human IgG (gamma chain specific) antibodies (Sera-Lab-Ltd., Crawley Down, UK) at 1 mg per well. After washing with PBST (phosphate buffered saline containing 0.02% Tween 20x (pH7.5)), 100ml of culture medium from the wells containing transfectants was added to each microtitre well for 1h at 37°C. The wells were then emptied, washed with PBST and either peroxidase-conjugated goat anti-human IgG or peroxidase-conjugated goat anti-human kappa constant region antibodies (both obtained from Sera-Lab Ltd., Crawley Down, UK) were added at 100 ng per well. Plates were then incubated at 37°C for 1h. The wells were then emptied and washed with PBST. 340 mg/ml o-phenylenediamine in 50mM sodium citrate, 50mM sodium phosphate (pH 5.0) and 0.003% (v/v) H_2O_2 were added at 200ml per well. Reactions were stopped after 1 to 5 min by the addition of 12.5% sulphuric acid at 50 ml per well. The absorbance at 492 nm was then measured spectrophotometrically.

- 23 -

The humanised antibody HuRSV19VH/VK, secreted from transfected cell lines cotransfected with pHuRSVVH and pHuRSVVK, was purified on Protein-A agarose columns (Boehringer Mannheim, Lewes, UK) and tested for binding to RSV virus in an ELISA assay. Antigen consisted of calf kidney (CK) cells infected with RSV (A2 strain of RSV obtained from a child in Australia and described by Lewis et al., *Med. J. Australia*, 48, 932-933 (1961)) and treated with 0.5% (v/v) NP40 detergent to yield a cell lysate. A control cell lysate was similarly prepared using uninfected CK cells. Microtitre plate wells were coated with either infected or control cell lysate. Antigen coated plates were blocked with PBST for 1 hour at 37°C, washed with PBST, and thereafter humanised antibody was applied (i.e., HuRSV19VH/VK). After 1 hour at 37°C, the wells were emptied, washed with PBST and 200 ng goat anti-human IgG antibodies (Sera Lab-Ltd., Crawley Down, UK) added per well. After 1 hour at 37°C, the wells were emptied, washed with PBST and 200ml of a 1:1000 dilution of horseradish peroxidase conjugated rabbit anti-goat IgG antibodies (Sigma-Poole, UK) were added. After 1 hour at 37°C, the wells were emptied and washed with PBST. To each well was added 200ml substrate buffer (340mg/ml α -phenylenediamine in 50mM sodium citrate, 50mM sodium phosphate (pH 5.0) and 0.003% (v/v) H₂O₂). Reactions were stopped by the addition of 50ml 12.5% sulphuric acid. The absorbance at 492 nm was then measured. Antibody HuRSVVH/VK bound to RSV although with an affinity less than the murine RSV19 antibody.

EXAMPLE 2-PRODUCTION OF HIGH AFFINITY ANTIBODIES SPECIFIC FOR RSV BY A METHOD DESIGNED TO ACHIEVE MINIMAL VARIABLE REGION FRAMEWORK MODIFICATIONS GIVING RISE TO HIGH AFFINITY BINDING

The method of this invention involves the following order of steps of alteration and testing:

- 24 -

1. Individual framework amino acid residues which are known to be critical for interaction with CDRs are compared in the primary antibody and the altered CDR-replacement antibody. For example, heavy chain amino acid residue 94 (Kabat numbering- see Kabat et al., cited above) is compared in the primary (donor) and altered antibodies. An arginine residue at this position is thought to interact with the invariant heavy chain CDR aspartic acid residue at position 101.

10

If amino acid 94 comprises arginine in the framework of the primary antibody but not in the framework of the altered antibody, then an alternative heavy chain gene comprising arginine 94 in the altered antibody is produced. In the reverse situation whereby the altered antibody framework comprises an arginine residue at position 94 but the primary antibody does not, then an alternative heavy chain gene comprising the original amino acid at position 94 is produced. Prior to any further analysis, alternative plasmids produced on this basis are tested for production of high affinity altered antibodies.

20

2. Framework amino acids within 4 residues of the CDRs as defined according to Kabat (see Kabat et al., cited above) are compared in the primary antibody and altered CDR-replacement antibody. Where differences are present, then for each region (e.g., upstream of VHCDR1) the specific amino acids of that region are substituted for those in the corresponding region of the altered antibody to provide a small number of altered genes. Alternative plasmids produced on this basis are then tested for production of high affinity antibodies.

30

3. Framework residues in the primary and altered CDR-replacement antibodies are compared and residues with major differences in charge, size or hydrophobicity are highlighted. Alternative plasmids are produced on this basis with the individual

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- 25 -

highlighted amino acids represented by the corresponding amino acids of the primary antibody and such alternative plasmids are tested for production of high affinity antibodies.

- 5 The method is exemplified by the production of a high affinity altered antibody derivative of HuRSVVH/VK (See, Example 1) specific for RSV. Comparison of VH gene sequences between RSV19VH and pHuRSV19VH (See, Figure 5) indicates that 3 out of 4 amino acid differences occur between amino acids 27 to 30 and
10 between amino acids 91 to 94. Thus, pHuRSV19VHNIK and pHuRSV19VHFNS were produced with framework amino acids 27 to 30 and 91 to 94 in the former, and amino acids 91 and 94 in the latter, represented as in the primary RSV19VH. Using oligonucleotide site directed mutagenesis as described in Example
15 1, the following oligonucleotides were used for mutagenesis of the HuRSV19VH gene in M13:

pHuRSV19VHNIK - 5'ATATAGTAGTCTTTAATGTTGAAGCCAGACA3'

pHuRSV19VHFNS - 5'CTCCCCCATGAATTACAGAAATAGACCG3'
20

- Humanised HuRSV19VHFNS/HuRSV19VK antibody was tested in an ELISA assay as detailed in Example 1 for analysis of binding to RSV antigen prepared from detergent-extracted, virus-infected
25 cells. Figure 6 shows that the substitution of VH residues 91 to 94 in HuRSV19VH/VK with VH residues from mouse RSV19VH partially restored antigen binding levels. Additional analysis of HuFNS binding properties was performed using an ELISA assay in which intact Type A RS virus (Long strain) was used as the
30 antigen. The data from such additional analysis (as shown in Figure 6A) show that there is little if any difference between the ability of the RSV19 and HuRSV19VHFNS/HuRSV19VK antibodies to bind to intact, non-denatured RS virus. This additional analysis also showed detectable binding of HuRSV19VH/VK to intact virus,
35 although of a much lower magnitude than was seen with either

- 26 -

RSV19 or HuRSV19VHFNS/HuRSV19VK. Thus, the data from this additional analysis suggests that the affinity for the native antigen was restored in the HuRSV19VHFNS/HuRSV19VK mAb. Specificity of HuRSV19VHFNS/HuRSV19VK for RSV F protein was shown by conventional Western blot analysis using a truncated soluble F protein construct expressed in CHO cells.

EXAMPLE 3-SPECIFICITY AND BIOLOGICAL ACTIVITY OF AN ALTERED ANTIBODY SPECIFIC FOR RSV.

10

In order to ascertain the potential clinical usefulness of a humanised antibody specific for RSV, an immunofluorescence analysis of binding to 24 RSV clinical isolates was undertaken. The isolates were obtained from children during the winter of 1983-84 by the Bristol Public Health Laboratory (Bristol, England) and represented both of the major subgroups of RSV. 13 isolates were serotyped as subgroup A and 11 isolates as subgroup B. HeLa or MA104 cells infected with RSV isolates were grown in tissue culture. When the cells showed evidence of cytopathic effect, 20 ml of 0.02% (w/v) disodium EDTA (ethylenediaminetetra-acetic acid) (BDH Chemicals Ltd., Poole, UK) in PBS and 3ml of 0.25% (w/v) trypsin in PBS were added and the cell suspension spotted into wells of PTFE-coated slides (polytetrafluoroethylene coated slides) (Hendley, Essex, UK). After 3 hours at 37°C, the slides were dried and fixed in 80% acetone. Cells were overlaid with monoclonal antibody (i.e., either humanised antibody, HuRSV19VHFNS/HuRSV19VK, or the murine antibody RSV19) for 1 hour at room temperature. After extensive washing, either fluorescein-conjugated rabbit anti-mouse IgG (Nordic Laboratories-Tilburg, The Netherlands) or fluorescein-conjugated goat anti-human IgG1 (Southern Biotechnology, Birmingham, Alabama, USA) was added, and the incubation was repeated. After further washing, cells were mounted in glycerol and examined under UV light.

- 27 -

Table I shows the results of comparative immunofluorescence for the humanised antibody, HuRSV19VHFNS/HuRSV19VK, and the murine antibody RSV19. This data indicates that 100% of clinical isolates are recognised by both the humanised and murine
5 antibodies. Such data demonstrates that the humanised antibody has the potential for recognition of most clinical isolates comprising both of the major RSV subgroups.

TABLE IBinding of Humanised Anti-RSV to Clinical Isolates

<u>Isolate Number</u>	<u>Extent of Fluorescence*</u>	
	<u>HuRSV19VHFNS/HuRSV19VK</u>	<u>Murine RSV19</u>
<u>Subgroup A</u>		
V818	++++	++++
V795	++++	++++
V00401	++	+++
V00214	+	++
V00764	++	+++
V743	++	+++
V316	++	++
V369	++++	++++
V1249	+++	+++
V04692	+++	+++
V1248	+	+
V01232	++	++
V729	+	++
<u>Subgroup B</u>		
V00634	+	++
V4715	++	+++
V00463	+	++
V4712	++	++
V00165	++	++
V00422	++	++
V837	+++	+++
V00900	++	++
4677	+++	+++
4424	++	++
V01231	+	+

* +, ++, +++ and ++++ refer to relative numbers of fluorescing cells observed and represent the proportion of cells infected

- 29 -

The humanised antibody, HuRSV19VHFNS/HuRSV19VK, was next tested for biological activity in vitro in a fusion inhibition assay. A suspension of MA104 cells was infected with RSV at an m.o.i. (multiplicity of infection) of 0.01 PFU (plaque forming units) per cell. After 1 hour at 37°C, 2ml of cells at 10^5 /ml were distributed to glass coverslips in tubes. After a further 24 hours at 37°C, the culture medium was replaced by medium containing dilutions of humanised antibody, HuRSV19VHFNS/HuRSV19VK. 24 hours later, coverslip cultures were fixed in methanol for 10 minutes and stained with May Grunwald stain (BDH Chemicals Ltd., Poole, UK). Table II shows the effect of increasing concentrations of HuRSV19VHFNS/HuRSV19VK in inhibiting the frequency of giant cells. The data represented in the following Table II demonstrates the biological activity of the humanised antibody

HuRSV19VHFNS/HuRSV19VK in inhibiting Type A RSV induced cell fusion. It should be noted that additional studies showed that the fusion inhibition titres for RSV19 versus HuRSV19VHFNS/HuRSV19VK were comparable, providing additional evidence that affinity for the native viral antigen was fully restored in HuRSV19VHFNS/HuRSV19VK. The humanized antibody HuRSV19VHFNS/HuRSV19VK has also been shown, (using methodology analogous to that utilized above for showing inhibition of Type A RSV induced cell fusion), to exhibit a dose dependent inhibition of Type B RSV (strain 8/60) induced giant cell fusion.

- 30 -

TABLE IIInhibition of RSV Induced Cell Fusion by Humanised Anti-RSV

Concentration of HuRSV19VHFNS/HuRSV19VK (ug/ml)	Number of Giant Cells*	Average number of Nucleii
100	44	4.5
50	71	4.0
25	40	3.8
12.5	67	
6.3	89	
3.1	87	
1.6	164	
0.8	201	
0.4	292	
0.2	219	
0	239,259	14,13.5
0 (no virus)	10	

* Scored as the number of cells with 2 or more nucleii in 20 fields with a 25x objective microscope lens

- 31 -

- The humanised antibody, HuRSV19VHFNS/HuRSV19VK was next tested for biological activity *in vivo* in an RSV-mouse infection model. BALB/c mice (obtained from Charles Rivers: specific pathogen free category 4 standard) were challenged intranasally
- 5 with 10^4 PFU of the A2 strain of human RSV (as described by Taylor et al., Infection and Immunity, 43 (1984) p649-655). Groups of mice were administered with 25mg of humanised antibody either one day prior to virus infection or 4 days following infection.
- 10 Administration of antibody was either by the intranasal (i.n.) or intraperitoneal (i.p.) routes. 5 days after RSV infection, mice were sacrificed and lungs were assayed for RSV PFU (see, Taylor et al., Infection and Immunity, 43 (1984) p649-655). The data in the following Table III shows that HuRSV19VHFNS/HuRSV19VK at a
- 15 single dose of 25mg per mouse is extremely effective in prevention and treatment of RSV infection.

TABLE IIIPrevention and Treatment of RSV Infection in Mice by Humanised Anti-RSVAntibody Treatment

<u>Day*</u>	<u>Route*</u>	<u>log₁₀ PFU per gram of lung⁻</u>
-1	i.p.	<1.7 <1.7 <1.7 <1.7 <1.7
-1	i.n.	<1.7 <1.7 <1.7 <1.7 <1.7
+4	i.p.	<1.7 <1.7 1.7 <1.7
+4	i.n.	<1.7 1.7 <1.7 1.7 <1.7
No antibody		4.47 4.32 4.64 4.61 4.55

* -1 refers to administration of HuRSV19VHFNS/HuRSV19VK antibody 1 day prior to RSV infection, +4 refers to administration of antibody 4 days post infection

+ i.p. - intraperitoneal, i.n. - intranasal

- virus PFU is expressed as the virus titre from dilutions of 10%, (w/v) lung homogenates (see Taylor et al., loc. cit.) adjusted to PFU per gram of lung. <1.7 log₁₀ PFU per gram means that no virus was detected in the starting dilution of lung homogenate 10%.

- 33 -

HuRSV19VHFNS/HuRSV19VK was also shown to be active in vivo when administered prophylactically to mice challenged with Type B RSV (strain 8/60) using methodology similar to that described above. In addition, the humanized antibody HuRSV19VH/VK was
5 also shown to be active in vivo when administered prophylactically to mice challenged with Type B RSV (strain 8/60) using methodology similar to that described above.

This invention also relates to a method of preventing human RSV
10 infection in a human in need thereof which comprises administering to such human an effective, human RSV infection inhibiting dose of an altered antibody of this invention for which RSV19 or RSV20 was the donor monoclonal antibody.

15 This invention also relates to a method of therapeutically treating human RSV infection in a human in need thereof which comprises administering to such human an effective, human RSV infection therapeutic dose of an altered antibody of this invention for which RSV19 or RSV20 was the donor monoclonal antibody.

20 To effectively prevent RSV infection in a human, one dose of approximately 1 mg/kg to approximately 20 mg/kg of an altered antibody of this invention for which RSV19 or RSV20 was the donor monoclonal antibody, such as HuRSV19VH/VK or
25 HuRSV19VHFNS/HuRSV19VK should be administered parenterally, preferably i.v. (intravenously) or i.m. (intramuscularly); or one dose of approximately 200 ug/kg to approximately 2 mg/kg of such antibody should be administered i.n. (intranasally). Preferably, such dose should be repeated every six
30 (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April). Alternatively, at the beginning of the RSV season, one dose of approximately 5 mg/kg to approximately 100 mg/kg of an altered antibody of this invention for which RSV19 or RSV20 was the donor
35 monoclonal antibody, such as HuRSV19VH/VK or

- 34 -

HuRSV19VHFNS/HuRSV19VK, should be administered i.v. or i.m. or one dose of approximately 0.5 mg/kg to approximately 10 mg/kg of such antibody should be administered i.n.

To effectively therapeutically treat RSV infection in a human, one
5 dose of approximately 2 mg/kg to approximately 20 mg/kg of an altered antibody of this invention for which RSV19 or RSV20 was the donor monoclonal antibody, such as HuRSV19VH/VK or HuRSV19VHFNS/HuRSV19VK should be administered parenterally., preferably i.v. or i.m.; or approximately 200 ug/kg to
10 approximately 2 mg/kg of such antibody should be administered i.n. Such dose may, if necessary, be repeated at appropriate time intervals until the RSV infection has been eradicated.

The altered antibodies of the invention may also be administered by
15 inhalation. By "inhalation" is meant intranasal and oral inhalation administration. Appropriate dosage forms for such administration, such as an aerosol formulation or a metered dose inhaler, may be prepared by conventional techniques. For example, to prepare a composition for administration by inhalation, for an aerosol
20 container with a capacity of 15-20 ml: Mix 10 mg of an altered antibody of this invention with 0.2-0.2% of a lubricating agent, such as polysorbate 85 or oleic acid, and disperse such mixture in a propellant, such as freon, preferably in a combination of (1,2 dichlorotetrafluoroethane) and difluorochloromethane and put into
25 an appropriate aerosol container adapted for either intranasal or oral inhalation administration. As a further example, for a composition for administration by inhalation, for an aerosol container with a capacity of 15-20 ml: Dissolve 10 mg of an altered antibody of this invention in ethanol (6-8 ml), add 0.1-0.2% of a
30 lubricating agent, such as polysorbate 85 or oleic acid; and disperse such in a propellant, such as freon, preferably a combination of (1,2 dichlorotetrafluoroethane) and difluorochloromethane, and put into an appropriate aerosol container adapted for either intranasal or oral inhalation administration.

- 35 -

The preferred daily dosage amount to be employed of an altered antibody of the invention to prophylactically or therapeutically treat RSV infection in a human in need thereof to be administered by inhalation is from about 0.1 mg to about 10 mg/kg per day).

5

Natural RSV infections have been reported in cattle, goats, sheep and chimpanzees. Thus, for example, utilizing the methodology described above, an appropriate mouse antibody could be "bovinized", and appropriate framework region residue alterations could be effected, if necessary, to restore specific binding affinity. Once the appropriate mouse antibody has been created, one of skill in the art, using conventional dosage determination techniques, can readily determine the appropriate dose levels and regimens required to effectively treat, prophylactically or therapeutically, bovine RSV infection.

10
15

Examples 1-3 show that altered antibodies for prevention and treatment of infection can be produced with variable region frameworks potentially recognised as "self" by recipients of the altered antibody. Minor modifications to the variable region frameworks can be implemented to effect large increases in antigen binding without appreciable increased immunogenicity for the recipient. Such altered antibodies can effectively prevent and eradicate infection.

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Thus the present invention provides an altered antibody in which complementarity determining regions (CDRs) in the heavy or light chain variable domains have been replaced by analogous parts of CDRs from a different source resulting in antibodies possessing the combination of properties required for effective prevention and treatment of infectious disease in animals or man. Suitably, the entire CDRs have been replaced. Preferably, the variable domains in both heavy and light chains have been altered by CDR replacement. Typically, the CDRs from a mouse antibody are grafted onto the framework regions of a human antibody. The

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- 36 -

altered antibody preferably has the structure of a natural antibody or a fragment thereof.

A preferred antibody is one directed against respiratory syncytial virus (RSV), preferably one specific for the fusion (F) protein of RSV. A particularly preferred antibody of this kind has the following N-terminal variable domain amino acid sequences (see the Amino Acid Shorthand Table immediately following) in its heavy and light chains:

10

heavy:

15

QVQLQESGPGGLVRPSQTL~~SLT~~CTVSGFT
~~FS~~(or ~~NIK~~)DYMHWRQPPGRGLEWIGWIDPEN
 DDVQYAPKFQGRVTMLVDTSKNQFSLRLSSVTAAD
 TAVYCAR(or ~~FCNS~~)WGSDFDHWGQGT~~TV~~TVSS

light:

20

DIQLTQSPSSLSASVGDRVTITCRSSQTLVHTDGNTY
 LEWYQQKPGAPKLLIYRVSNRFSGVPSRFSGSGSGT
 DFTFTISLQPEDLATYYCQSHLPRTFGQGTKVEIK

Table Amino Acid Shorthand

25	Amino Acid	Three-letter abbreviation	One-letter symbol
	Alanine	Ala	A
	Arginine	Arg	R
30	Asparagine	Asn	N
	Aspartic acid	Asp	D
35	Cysteine	Cys	C
	Glutamine	Gln	Q
	Glutamic acid	Glu	E

- 37 -

	Glutamic acid	Glu	E
	Glycine	Gly	G
5	Histidine	His	H
	Isoleucine	Ile	I
10	Leucine	Leu	L
	Lysine	Lys	K
	Methionine	Met	M
15	Phenylalanine	Phe	F
	Proline	Pro	P
20	Serine	Ser	S
	Threonine	Thr	T
	Tryptophan	Trp	W
25	Tyrosine	Tyr	Y
	Valine	Val	V

30 It will be understood by those skilled in the art that such an altered antibody may be further altered by changes in variable domain amino acids without necessarily affecting the specificity of the antibody for the fusion (F) protein of RSV, and it is anticipated that even as many as 25% of heavy and light chain amino acids may be

35 substituted by other amino acids either in the variable domain frameworks or CDRs or both. Such altered antibodies can be effective in prevention and treatment of respiratory syncytial virus (RSV) infection in animals and man.

40 The invention also includes a recombinant plasmid containing the coding sequence of the altered antibody of the invention, and a

- 38 -

mammalian cell-line transfected with a recombinant plasmid containing the coding sequence of the altered antibodies hereof. Such a vector is prepared by conventional techniques and suitably comprises DNA sequences encoding immunoglobulin domains including variable region frameworks and CDRs derived from a different source and a suitable promoter operationally linked to the DNA sequences which encode the altered antibody. Such a vector is transfected into a transfected mammalian cell via conventional techniques.

The invention further comprises a method for effecting minimal modifications within the variable region frameworks of an altered antibody necessary to produce an altered antibody with increased binding affinity comprising the following steps:

- (a) analysis of framework amino acids known to be critical for interaction with CDRs, and production and testing of altered antibodies where single framework amino acids have been substituted by the corresponding amino acids from the same source as the CDRs;
- (b) analysis of framework amino acids adjacent to CDRs, and production and testing of altered antibodies where one or more of the amino acids within 4 residues of CDRs have been substituted by the corresponding amino acids from the same source of the CDRs;
- (c) analysis of framework residues within the altered antibody, and production and testing of altered antibodies where single amino acids have been substituted by the corresponding amino acids with major differences in charge, size or hydrophobicity from the same source of CDRs.

The following Examples relate to the novel RSV F protein epitope of the invention.

- 39 -

SPECIFIC RSV F PROTEIN EPITOPE

The following examples demonstrate that two monoclonals which protect and cure mice of *in vivo* infection by RSV recognize a linear epitope within the F protein of RSV (which linear epitope may be part of a conformational epitope) and which contains amino acid residues 417 to 438 of the F protein coding sequence including an essential arginine residue at position 429, or any immunoprotective portion thereof, such as, but not limited to amino acid residues 417-432 of the F protein coding sequence, and amino acid residues 422-438 of the F protein coding sequence. This novel epitope (which may be referred to herein as "epitope 417-438") is a suitable target for screening for other neutralizing epitopes, for protective and therapeutic agents against RSV, and in particular, for monoclonal antibodies against this epitope. Knowledge of this epitope enables one of skill in the art to define synthetic peptides which would be suitable as vaccines against RSV. Epitope 417-438 is also useful for generating monoclonal antibodies which will be useful in the treatment, therapeutic and/or prophylactic, of human RSV infection in humans.

The present invention also applies to the use of Fab fragments derived from monoclonal antibodies directed against such novel epitope as protective and therapeutic agents against *in vivo* infection by viruses, and particularly relates to the protection against RSV.

The invention also includes a recombinant plasmid containing the coding sequence of a monoclonal antibody generated against the 417-438 epitope, and a mammalian cell-line transfected with a recombinant plasmid containing such coding sequence. Such a vector is prepared by conventional techniques and suitably comprises DNA sequences encoding immunoglobulin domains including variable region frameworks and CDRs and a suitable promoter operationally linked to the DNA sequences which encode

- 40 -

the antibody. Such a vector is transfected into a mammalian cell via conventional techniques.

EXAMPLE 4

5

This example shows the production of murine monoclonal antibodies against the F protein of RSV which protect and cure mice of infection.

- 10 Murine monoclonal antibodies (mAbs) 19 and 20 were produced as follows. BALB/c mice (obtained from Charles Rivers-specific pathogen free) were inoculated intranasally (i.n.) on two occasions, 3 weeks apart, with 1×10^4 PFU of the A2 strain of human (H) RSV (described by Lewis et al., 1961, Med. J. Australia, 48, 932-933).
- 15 After an interval of 4 months, the mice were inoculated intraperitoneally (i.p.) with 2×10^7 PFU of the 127 strain of bovine (B) RSV (isolated at Institute for Animal Health, Compton, Near Newbury, Berks, England). Three days after inoculation, the immune splenocytes were fused with NS-1 myeloma cells (see,
- 20 Williams et al., 1977, Cell, 12, 663). The resulting hybridomas were screened for antibody to RSV by radioimmunoassay and immunofluorescence as described previously (Taylor et. al., 1984, Immunology, 52, 137-142), cloned twice on soft agar (as described by Kohler et. al., "Immunologic Methods", pp397-402, ed. I.
- 25 Lefkovitz & B. Perris, Academic Press), and the resulting cloned cells were inoculated into BALB/c mice to produce ascitic fluid as described previously (see, Taylor et al., 1984, Immunology, 52, 137-142).
- 30 The specificity of the mAbs for viral polypeptides was determined by radioimmune precipitation of (35 S)-methionine or (3 H)-glucosamine labelled RSV-infected cell lysates as described previously (see, Kennedy, et al., 1988, J. Gen Virol, 69, 3023-2032) and by immunoblotting (see, Taketa et al., 1985, Electrophoresis, 6,
- 35 492-497). The antigens used in immunoblotting were either Hep-2

- 41 -

cells (obtained from the American Type Culture Collection, Rockville, Maryland, USA) infected with the A2 strain of HRSV or primary calf kidney (CK) cells (produced at the Institute for Animal Health, Compton) infected with the 127 strain of BRSV. Uninfected
5 Hep-2 or CK cells were used as control antigens.

The immunoglobulin isotype of the mAbs was determined by immunodiffusion using a radial immunodiffusion kit (Serotec, Kidlington, Oxfordshire, UK).

10

The properties of mAbs 19 and 20 are shown in the following Table A.

TABLE A

Table A Properties of mAb 19 and mAb 20

mAb	Protein specificity			Ig class	ELISA titre ¹			% SFA ²	Complement lysis ³	Neut. titre ⁴	Fusion inhibition	Mouse protection ⁵
	RIPA	Western blot										
		Native	Reduced									
19	I ⁺	140K, 70K	46K	G2a	7.2	7.4	6.7	88	1.7	3.4	+	>3.8
20	I ⁺	140K, 70K	46K	G2a	>6.0	8.6	7.5	69	76	4.3	+	>3.8

¹Antibody titre, using HRSV strain A2 (subtype A), HRSV strain 8/60 (subtype B) and BRSV strain 127 as antigens in ELISA, expressed as log₁₀ titre.

²SFA = percent of HRSV strain A2 infected cells showing surface fluorescence.

³Percent specific chromium release from virus infected cells (bovine nasal mucosa cells persistently infected with BRSV) by 1/100 dilution of mAb and rabbit complement.

⁴50% plaque reduction titre expressed as log₁₀.

⁵Reduction in peak titre of RSV, strain A2 in lungs of mice given 100 µl of mAb intra peritoneally one day before intranasal challenge, expressed as log₁₀ pfu.

- 43 -

Immune precipitation of radiolabelled RSV (by the method of Brunda et al, (1977) J. Immunol. 119, 193-198) indicated that mAbs 19 and 20 recognized the fusion (F) glycoprotein. This was confirmed by a Western blot of non-reduced and reduced lysates of cells infected with RSV. The blots were probed with HRP-conjugated goat anti-mouse IgG (Kpl, Gaithersburg, Maryland, USA). mAbs 19 and 20 recognized the 140k F protein dimer and the 70K monomer present in the native F protein antigen and the 46K F1 fragment in antigen denatured by boiling in 2-mercaptoethanol. Both mAb 19 and 20 were identified as IgG2a, and their ELISA titres against the A2 and 8/60 strains of HRSV were similar to the ELISA titres against the 127 strain of BRSV, indicating that the epitopes recognized by these mAbs were conserved amongst strains of human and bovine RSV. Both mAb 19 and 20 neutralized RSV infectivity and inhibited the formation of multinucleated giant cells in MA104 cells infected with RSV. In contrast to mAb 19, mAb 20 lysed RSV-infected cells in the presence of rabbit complement. The failure of mAb 19 to lyse RSV-infected cells was not due to failure to bind to the surface of virus-infected cells since mAb 19 stained 88% of such cells. The failure of mAb 19 and complement to lyse virus-infected cells indicates that antibody and complement-mediated lysis is not important in the *in vivo* protection mediated by this antibody. The ability of mAbs 19 and 20 to protect against RSV infection was assessed by challenging mice i.n. with approximately 10^4 PFU of RSV 24 h after i.p. inoculation of mAbs 19 and 20. The lungs of untreated mice killed 5 days after challenge contained $5.5 \log_{10}$ PFU of RSV/g tissue whereas virus was not detected in the lungs of mice given either mAb 19 or 20.

EXAMPLE 5

This example describes methods of isolating mutants of RSV which are resistant to inhibition by mAbs 19 and 20 generated in Example 4.

- 44 -

- Mutant RS viruses refractory to neutralization by mAbs 19 and 20 were produced using a plaque reduction technique with the A2 strain of HRSV as follows. Confluent monolayers of CK cells, in a tissue culture flask, were infected with the A2 strain of HRSV at a MOI of 0.1. Starting 24 hours after infection and continuing for 3 to 5 days, the culture medium was replaced daily with fresh medium containing 10% mAb. Virus was harvested when a cytopathic effect was observed. Virus prepared in this way was mixed with an equal volume of either undiluted mAb 19 or 20, or medium alone for 1 hour at room temperature and inoculated onto CK monolayers in 35mm multi-well plates (Nunc, Kamstrup, Riskilde, Denmark). After 1 hour incubation at 37°C, the plates were overlaid with medium containing 0.25% agarose and 10% mAb or medium alone. Cultures were incubated at 37°C in 5% CO₂ in air for 7 days before adding the vital stain, 0.3% 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide in 0.15M NaCl, to the overlay to visualize virus plaques.
- Putative mutant viruses were removed in agar plugs from plates which contained single plaques, diluted in medium, mixed with an equal volume of mAbs 19 or 20 and inoculated onto CK monolayers in 35 mm multi-well plates as before. Putative mutant viruses were plaque picked again and inoculated into tubes containing coverslips of calf testes cells. After 4 to 6 days incubation, the coverslips were removed and stained with mAb 19 and 20 and FITC-labelled rabbit anti-mouse Ig (Nordic Labs, Tilburg, The Netherlands). As a positive control, coverslips were stained with polyclonal bovine antiserum to RSV (produced at Institute for Animal Health-Compton form a gnotobiotic calf hyperimmunised with RSV), and FITC-labelled rabbit anti-bovine Ig (obtained from Nordic Immunology, Tilburg, The Netherlands). RS viruses that failed to react by immunofluorescence to mAb 19 or 20 were classed as mutant viruses and were used to infect monolayers of Hep-2 cells to produce antigen for ELISA. Thus, 3 to 4 days after RSV infection,

- 45 -

cells were scraped into the medium, spun at 400 g for 5 mins, resuspended in distilled water, and treated with 0.5% (v/v) NP40 detergent to yield a cell lysate. A control cell lysate was made in a similar way using uninfected Hep-2 cells. The binding of a panel of

5 mAbs to the F protein of RSV to the mutant viruses was examined by ELISA. Microtitre plate wells were coated with 50 ul of either infected or control cell lysate overnight at 37°C, incubated with blocking buffer consisting of 5% normal pig serum in PBS and 0.05% Tween 20 for 1 h at room temperature and washed 5x with

10 PBS/TWEEN. Serial dilutions (three times) of the mAbs were added to the wells and the plates were incubated for 1 hour. After washing 5 times with PBS/Tween, HRP-conjugated goat anti-mouse IgG (Kpl, Gaithersburg, Maryland, USA), diluted 1:2000, was added to each well. After a final washing, bound conjugate was

15 detected using the substrate 3,3',5,5'-tetramethylbenzidine (TMB), (obtained from ICN Immunobiologicals, Illinois). Mutant viruses, selected for resistance to mAb 19, failed to react in ELISA with both mAbs 19 and 20. Similarly, mutant viruses selected for resistance to mAb 20 failed to react with mAbs 19 and 20. All other mAbs

20 tested reacted with the mutants to the same extent as to parent HRSV, strain A2. These results are illustrated in the following Table B.

TABLE B

Table B Binding of anti-F mAbs to antibody escape mutants of RSV.

mAb	Parent A2	Mutants selected with indicated mAb					
		19			20		
		C4848f	C4909/1	C4902/6	C4902Wa	C42902Wb	C4902Wc
1	+	+	+	+	+	+	+
2	+	+	+	+	÷	÷	÷
5	+	+	÷	÷	+	÷	÷
11	+	+	+	+	÷	÷	+
13	÷	+	+	÷	÷	÷	÷
14	+	+	+	÷	÷	÷	÷
16	+	÷	+	÷	+	÷	÷
17	+	+	÷	÷	+	÷	÷
18	+	+	+	+	+	÷	+
19	+	-	-	-	-	-	-
20	÷	-	-	-	-	-	-
21	÷	+	÷	÷	÷	÷	÷
B1	+	+	÷	÷	÷	+	÷
B2	+	+	÷	+	+	÷	÷
B3	+	+	+	+	+	÷	÷
B4	+	+	+	÷	+	÷	÷
B5	÷	+	÷	÷	÷	÷	÷
B6	+	÷	+	+	÷	÷	÷
B7	+	+	+	÷	+	÷	÷
B8	+	+	+	+	+	÷	÷
B9	+	+	+	÷	+	÷	÷
B10	+	+	÷	÷	+	÷	÷
7C2	+	+	+	÷	+	÷	÷
47F	+	+	+	÷	+	÷	÷

- 47 -

EXAMPLE 6

This example describes the identification of an amino acid sequence within the F protein which binds protective monoclonal antibodies and demonstrates that arginine 429 is essential for binding protective mabs to this amino acid sequence.

Poly(A)⁺ RNAs, isolated from cells infected with either the A2 strain of HRSV or each of the mutants described in Example 5, were used to sequence the F protein mRNA. These sequences were determined by the dideoxy method (cited above) using 5'-³²P-labelled oligonucleotide primers, synthesized according to the previously reported F-protein sequence of the Long strain of RSV (see, Lopez, et al., 1988, Virus Res. 10, 249-262), followed by a chase with terminal deoxynucleotide transferase (see, DeBorde, et al., 1986, Anal Biochem. 157, 275-282). Three mutants were selected with mAb 19 and three were selected with mAb 20. All such mutants showed a single transversion (C to G) at nucleotide 1298 compared with the parent A₂ strain. This nucleotide substitution changes the amino acid residue at position 429 of the F protein from arginine to serine. Since mAbs 19 reacted in Western blot with the F₁ subunit, it is likely that the antibody-binding site is determined by a linear sequence of contiguous amino acids in which residue 429 of the F₁ subunit plays an essential role.

Synthetic peptides corresponding to amino acids residues 417-432, 422-438, 417-438 and 421-450 of the F protein were examined for their ability to react with mAbs 19 and 20 in ELISA. mAbs 19 and 20 reacted with peptides 417-432 (F417), 417-438 and with 422-438 (F422) but not with peptide 431-450. The binding of mAb 19 to peptides 417-432 and 422-438 (2ug/well) either coated onto microtitre plate wells overnight at 37°C ("dry") or coated onto the wells for 1h at room temperature ("wet") is shown in Figure 7. It should be noted that mAb 20 gave essentially the same results.

Example 7

- 5 This example shows that Fab fragments derived from mAbs 19 and 20 can protect and treat mice infected by RSV.

mAbs 19 and 20 were purified from ascitic fluid using Protein A Sepharose (Pharmacia, Milton Keynes, United Kingdom).

10 Approximately 10 mg of purified mAb 19 and 20 were incubated with 0.5 ml of immobilized papain (Pierce-Oud-Beijerland, The Netherlands) for 5 h and overnight respectively at 37°C with constant mixing. The resulting Fab fragments were recovered on an immobilized Protein A column (Pierce). The purified IgG and

15 the papain cleaved fragments were analyzed by SDS-PAGE under reducing conditions. The purified IgG showed bands at 53,000d and 23,000d, corresponding to Ig heavy and light chains. The Protein A fractions containing Fab fragments showed bands at approximately 25,000d and the fraction containing the Fc

20 fragments showed 3 distinct bands corresponding to the heavy and light chains of the undigested IgG and also the Fc fragment at approximately 28,000d. The purified IgG and the papain cleaved fragments were evaluated for anti-RSV activity by ELISA with HRSV strain A2 infected and uninfected Hep-2 cells as antigen, and

25 HRP-goat anti-mouse Fab (Sigma Chemical Co., St. Louis, Mi, USA) and HRP-goat anti-mouse Fc (ICN ImmunoBiologicals, Illinois). The ELISA showed that the Fab fragments of mAbs 19 and 20 were not contaminated with undigested Ig. These data are illustrated in the following Table C.

TABLE C

Table C Prophylactic and therapeutic effects of Fab fragments on RSV infection in mice.

Antibody	ELISA titre (log ₁₀)		D5 RSV titre in lungs	
	Anti-Fc	Anti-Fab	mAb d-1	mAb d4
19	4.4	4.3	<1.7 (0/5)	<1.7 (0/4)
19 Fab	<2.0	4.6	<1.7 (0/5)	<1.7 (0/5)
None			4.6 ± 0.06	
20	5.1	5.1	<1.7 (0/5)	<1.7 (1/5)
20 Fab	<2.0	4.8	<1.7 (2/5)	<1.7 (2/5)
None			4.5 ± 0.08	

Antibody titre measured by ELISA using RSV/A2 and antigen

- 50 -

The concentration of antibody in undigested mAbs 19 and 20 were adjusted to give ELISA titres similar to those of the Fab fragments and examined for their ability to protect against RSV infection in BALB/c mice. Groups of 5 mice were inoculated i.n. with
5 undigested, purified mAb 19 or mAb 20 or Fab fragments (from mAb 19 or mAb 20) either 1 day before or 4 days after i.n. inoculation with approximately 10^4 PFU of the A2 strain of HRSV. Control mice were inoculated with HRSV only. Five days after virus challenge, mice were killed and the lungs assayed for RSV
10 PFU on secondary CK cells as described previously (see, Taylor et al., 1984, Infect Immun. 43, 649-655). Fab fragments of mAbs 19 and 20 were highly effective both in preventing RSV infection and in clearing an established infection.

15 This invention relates to the 417-438 epitope. This invention also relates to monoclonal antibodies generated against the 417-438 epitope. Such monoclonal antibodies are produced by conventional techniques and include, without limitation, murine monoclonal antibodies, human monoclonal antibodies, and bovine monoclonal
20 antibodies. Such monoclonal antibodies may comprise a complete antibody molecule (having full length heavy and light chains) or any fragment thereof, such as the Fab or (Fab')₂ fragment, a light chain or heavy chain dimer, or any minimal recombinant fragment thereof such as an Fv or a SCA (single-chain antibody) or any other
25 molecule with the same specificity as the monoclonal antibody.

This invention also relates to a pharmaceutical composition comprising a monoclonal antibody generated against the 417-438 epitope and a pharmaceutically acceptable carrier or diluent.

30 This invention also relates to a method of preventing human RSV infection in a human in need thereof which comprises administering to such human an effective, human RSV infection inhibiting dose of a monoclonal antibody generated against the 417-
35 438 epitope.

- 51 -

This invention also relates to a method of therapeutically treating human RSV infection in a human in need thereof which comprises administering to such human an effective, human RSV infection therapeutic dose of a monoclonal antibody generated against the 417-438 epitope.

To effectively prevent RSV infection in a human, one dose of approximately 1 mg/kg to approximately 20 mg/kg of a monoclonal antibody generated against the 417-438 epitope should be administered parenterally, preferably i.v. (intravenously) or i.m. (intramuscularly); or one dose of approximately 200 ug/kg to approximately 2 mg/kg of such antibody should be administered i.n. (intranasally). Preferably, such dose should be repeated every six (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April). Alternatively, at the beginning of the RSV season, one dose of approximately 5 mg/kg to approximately 100 mg/kg of a monoclonal antibody generated against the 417-438 epitope should be administered i.v. or i.m. or one dose of approximately 0.5 mg/kg to approximately 10 mg/kg of such antibody should be administered i.n.

To effectively therapeutically treat RSV infection in a human, one dose of approximately 2 mg/kg to approximately 20 mg/kg of a monoclonal antibody generated against the 417-438 epitope should be administered parenterally., preferably i.v. or i.m.; or approximately 200 ug/kg to approximately 2 mg/kg of such antibody should be administered i.n. Such dose may, if necessary, be repeated at appropriate time intervals until the RSV infection has been eradicated.

A monoclonal antibody generated against the 417-438 epitope may also be administered by inhalation. By "inhalation" is meant intranasal and oral inhalation administration. Appropriate dosage

- 52 -

forms for such administration, such as an aerosol formulation or a metered dose inhaler, may be prepared by conventional techniques. For example, to prepare a composition for administration by inhalation, for an aerosol container with a capacity of 15-20 ml: Mix
5 10 mg of a monoclonal antibody generated against the 417-438 epitope with 0.2-0.2% of a lubricating agent, such as polysorbate 85 or oleic acid, and disperse such mixture in a propellant, such as freon, preferably in a combination of (1,2 dichlorotetrafluoroethane) and difluorochloromethane and put into an appropriate aerosol
10 container adapted for either intranasal or oral inhalation administration. As a further example, for a composition for administration by inhalation, for an aerosol container with a capacity of 15-20 ml: Dissolve 10 mg of a monoclonal antibody generated against the 417-438 epitope in ethanol (6-8 ml), add 0.1-
15 0.2% of a lubricating agent, such as polysorbate 85 or oleic acid; and disperse such in a propellant, such as freon, preferably a combination of (1,2 dichlorotetrafluoroethane) and difluorochloromethane, and put into an appropriate aerosol container adapted for either intranasal or oral inhalation
20 administration.

The preferred daily dosage amount to be employed of a monoclonal antibody generated against the 417-438 epitope to prophylactically or therapeutically treat RSV infection in a human in need thereof to
25 be administered by inhalation is from about 0.1 mg to about 10 mg/kg per day.

- 53 -

CLAIMS

What is claimed is:

- 5 1. An altered antibody in which at least parts of the
complementarity determining regions (CDRs) in the light and/or
heavy variable domains of an acceptor monoclonal antibody have
been replaced by analagous parts of CDRs from one or more donor
monoclonal antibodies, and in which there may or may not have
10 been minimal alteration of the acceptor monoclonal antibody light
and/or heavy variable domain framework region in order to retain
donor monoclonal antibody binding specificity, wherein such donor
antibodies have specificity for a particular microorganism.
- 15 2. The antibody of Claim 1 wherein the microorganism is human
respiratory syncytial virus (RSV).
3. The antibody of Claim 2 wherein the donor antibody is directed
against the fusion (F) protein of RSV.
- 20 4. The antibody of Claim 2 wherein the donor antibody is directed
against epitope 417-438.
5. The antibody of Claim 2 which has the following N-terminal
25 variable domain amino acid sequences in its heavy and light chains:

heavy:

30 QVQLQESGPGGLVRPSQTL~~SLT~~CTVSGFT
ES(or ~~NIK~~)DY~~Y~~MHWVRQPPGRGLEWIGWIDPEN
DDVQYAPKFQGRVTMLVDTSKNQFSLRLSSVTAAD
TAVYYCAR(or ~~FCNS~~)WGSDFDHWGQGTTVTVSS

- 54 -

light

QLTQSPSSLSASVGDRVTTTCRSSQTLVHTDGNTYL
 EWYQQKPGAPKLLIYRVSNRFSGVPSRFSGSGSGTD
 FTFITISLQPEDATYYCQSHLPRTFGQGTKVEIK

5

6. The antibody of Claim 2 wherein the donor monoclonal antibody is RSV19.

7. The antibody of Claim 2 wherein the donor monoclonal antibody is RSV20.

8. The antibody of Claim 6 which is HuRSV19VH/VK.

9. The antibody of Claim 6 which is
 HuRSV19VHFNS/HuRSV19VK.

10. The antibody of Claim 1 which is a Fab fragment or a (Fab')₂ fragment.

11. A pharmaceutical composition comprising the altered antibody of Claim 1 and a pharmaceutically acceptable carrier or diluent.

12. The composition of Claim 11 wherein the microorganism is human RSV.

25

13. The composition of Claim 11 wherein the donor antibody is directed against the fusion (F) protein of RSV.

14. The composition of Claim 11 wherein the donor antibody is directed against epitope 417-438.

15. The composition of Claim 11 wherein the altered antibody has the following N-terminal variable domain amino acid sequences in its heavy and light chains:

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- 55 -

heavy:

QVQLQESGPGGLVRPSQTLSTCTVSGFT
 ES(or NIK)DYMHVWRQPPGRGLEWIGWIDPENDD
 VQYAPKFQGRVTMLVDTSKNQFSLRLSSVTAADTA
 5 VYYCAR(or FCNS)WGSDFDHWGQGTTV TVSS

light:

IQLTQSPSSLSASVGDRVTTTCRSSQTLVHTDGNTYL
 10 EWYQQKPGAPKLLIYRVSNRFSGVPSRFSGSGSGTD
 FTFTISLQPEDLATYYCQSHLPRTFGQGTKVEIK

16. The composition of Claim 11 wherein the donor monoclonal
 15 antibody is RSV19.

17. The composition of Claim 11 wherein the donor monoclonal
 antibody is RSV20.

20 18. The composition of Claim 16 wherein the altered antibody is
 HuRSV19VH/VK.

19. The composition of Claim 16 wherein the altered antibody is
 HuRSV19VHFNS/HuRSV19VK.

25 20.. The composition of Claim 10 wherein the altered antibody is a
 Fab fragment or a (Fab')₂ fragment.

30 21. A method of preventing human RSV infection in a human in
 need thereof which comprises administering to such human, an
 effective, human RSV infection inhibiting dose of the altered
 antibody of any of Claims 4, 5, 6, 7, 8, and 9.

35 22. The method of Claim 21 wherein one dose of approximately 1
 mg/kg to approximately 20 mg/kg of the altered antibody is

- 56 -

22. The method of Claim 21 wherein one dose of approximately 1 mg/kg to approximately 20 mg/kg of the altered antibody is administered parenterally, preferably i.v. (intravenously) or i.m. (intramuscularly).

5

23. The method of Claim 21 wherein one dose of approximately 200 ug/kg to approximately 2 mg/kg of the altered antibody is administered i.n. (intranasally).

24. The method of Claim 22 wherein the dose is repeated every six (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April).

25. The method of Claim 23 wherein the dose is repeated every six (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April).

15

26. The method of Claim 21 wherein, at the beginning of the RSV season, one dose of approximately 5 mg/kg to approximately 100 mg/kg of the altered antibody is administered i.v. or i.m., or one dose of approximately 0.5 mg/kg to approximately 10 mg/kg of such antibody is administered i.n.

20

27. The method of Claim 21 wherein the altered antibody is HuRSV19VHFNS/HuRSV19VK.

25

28. The method of Claim 21 wherein the altered antibody is HuRSV19VH/VK.

29. A method of therapeutically treating human RSV infection in a human in need thereof which comprises administering to such human an effective, human RSV infection therapeutic dose of the altered antibody of any of Claims 4, 5, 6, 7, 8, and 9.

30

- 57 -

30. The method of Claim 29 wherein one dose of approximately 2 mg/kg to approximately 20 mg/kg of the altered antibody is administered parenterally., preferably i.v. or i.m.
- 5 31. The method of Claim 29 wherein approximately 200 ug/kg to approximately 2 mg/kg of the altered antibody is administered i.n.
32. A monoclonal antibody generated against Epitope 417-438.
- 10 33. A pharmaceutical composition comprising the monoclonal antibody of Claim 32 and a pharmaceutically acceptable carrier or diluent.
34. A method of preventing human RSV infection in a human in need thereof which comprises administering to such human an
- 15 effective, human RSV infection inhibiting dose of the monoclonal antibody of Claim 32.
35. The method of Claim 34 wherein one dose of approximately 1 mg/kg to approximately 20 mg/kg of the monoclonal antibody is
- 20 administered parenterally, preferably i.v. (intravenously) or i.m. (intramuscularly).
36. The method of Claim 34 wherein one dose of approximately 200 ug/kg to approximately 2 mg/kg of the monoclonal antibody is
- 25 administered i.n. (intranasally).
37. The method of Claim 35 wherein such dose is repeated every six (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April).
- 30 38. The method of Claim 36 wherein such dose is repeated every six (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April).

- 58 -

39. The method of Claim 34 wherein, at the beginning of the RSV season, one dose of approximately 5 mg/kg to approximately 100 mg/kg of the monoclonal antibody is administered i.v. or i.m., or one dose of approximately 0.5 mg/kg to approximately 10 mg/kg of such antibody is administered i.n.

40. A method of therapeutically treating human RSV infection in a human in need thereof which comprises administering to such human an effective, human RSV infection therapeutic dose of the monoclonal antibody of Claim 32.

41. The method of Claim 40 wherein one dose of approximately 2 mg/kg to approximately 20 mg/kg of the monoclonal antibody is administered parenterally, preferably i.v. or i.m.

42. The method of Claim 40 wherein approximately 200 ug/kg to approximately 2 mg/kg of the antibody is administered i.n.

43. The monoclonal antibody of Claim 32 which is a Fab fragment.

44. The method of Claim 34 wherein the monoclonal antibody is administered by inhalation.

45. The method of Claim 40 wherein the monoclonal antibody is administered by inhalation.

46. The method of Claim 21 wherein the altered antibody is administered by inhalation.

47. The method of Claim 29 wherein the altered antibody is administered by inhalation.

48. A method for effecting minimal modifications within the variable region frameworks of an altered antibody necessary to produce an altered antibody with increased binding affinity comprising the following steps:

- 59 -

(a) analysis of framework amino acids known to be critical for interaction with CDRs, and production and testing of altered antibodies where single framework amino acids have been substituted by the corresponding amino acids from the same source as the CDRs;

(b) analysis of framework amino acids adjacent to CDRs, and production and testing of altered antibodies where one or more of the amino acids within 4 residues of CDRs have been substituted by the corresponding amino acids from the same source of the CDRs;

(c) analysis of framework residues within the altered antibody, and production and testing of altered antibodies where single amino acids have been substituted by the corresponding amino acids with major differences in charge, size or hydrophobicity from the same source of CDRs.

49. A recombinant plasmid containing the coding sequence of the altered antibody of Claim 1.

50. A mammalian cell-line transfected with the recombinant plasmid of Claim 49.

51. A recombinant plasmid containing the coding sequence of the monoclonal antibody of Claim 32.

52. A mammalian cell line transfected with the recombinant plasmid of Claim 51.

1 / 8

^q v ^q l ^q e/q ^s g t e l e r s g a s v k l s c t a s g
 CAGGTCAGCTGCAGSAGTCWGGGACAGAGCTTGAGAGGTCAGGGGCTCAGTCAAGTTGTCTGCACAGCTTCTGGC
^f n ⁱ k ^d y y m ^h w m k q r p d q g l e w l g ^w l ^d
 TTCAACATTAAAGACTACTATATGCACCTGGATGAAGCAGAGGCTGACCAGGGCTGGAGTGGATTGGATTGA
^p e n d d v q y a p k f q g k a t m t a d t s s n t
 TCCTGAGAAATGATGATGTCATAATGCCCCGAAGTTCAGGGCAAGGCCACTATGACTGCAGACACGTCTCCAACAC
^a y l q l t s l t f e d t a v y f c n s ^w g s d f d
 AGCCTACCTGCAGCTCACCGCCTGACATTTGAGGACACTGCCGTCTATTCTGTAAATTCATGGGGGAGTGACITTGA
^h w g q g t t v t v s s
 CCAGTGGGGCCCAAGGGACCCACGGTCACCGTCTCTCTCA

FIG. 1

2 / 8

d i q l t q s p l s l p v t l g d q a s i s c r s s q
GACATTCAGCTGACCCAGTCCTCCACTCTCCCTGCCCTGTCACCTCTGGAGATCAAGCCTCCATCTCTTGCAGATCTAGTCAG

t l v h t d g n t y l e w f l q k p g q s p k l l i y
ACCTTGTACATACIGATGGAAACACCTATTTAGAAATGGTTTCTGCAGAAACCAGGCCAGTCTCCAAAGCTCCCTGATCTAC

r v s n r f s g v p d r f s g s g t d f t l k i s
AGAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTTCAGTGGCAGTGGATCAGGGACAGATTTTCACACTCAAGATCAGC

r v e a e d l g v y f c f a g s h l p r t f g g g t k
AGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTTTCAGGTTTCACATCTTCTCGGACGTTTCGGTGGAGGCCAAG

l e i
CTGGAGATCTAAC

FIG. 2

3 / 8

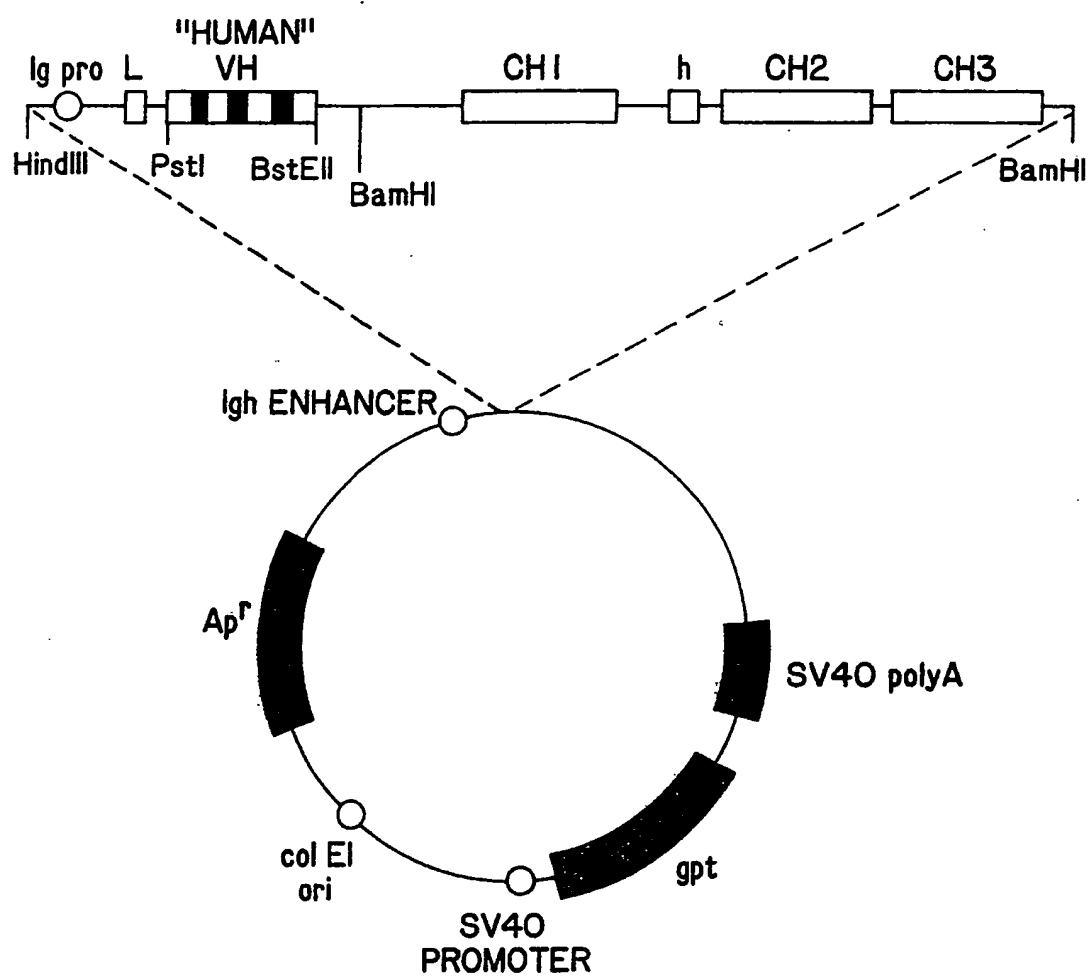


FIG. 3

SUBSTITUTE SHEET

4 / 8

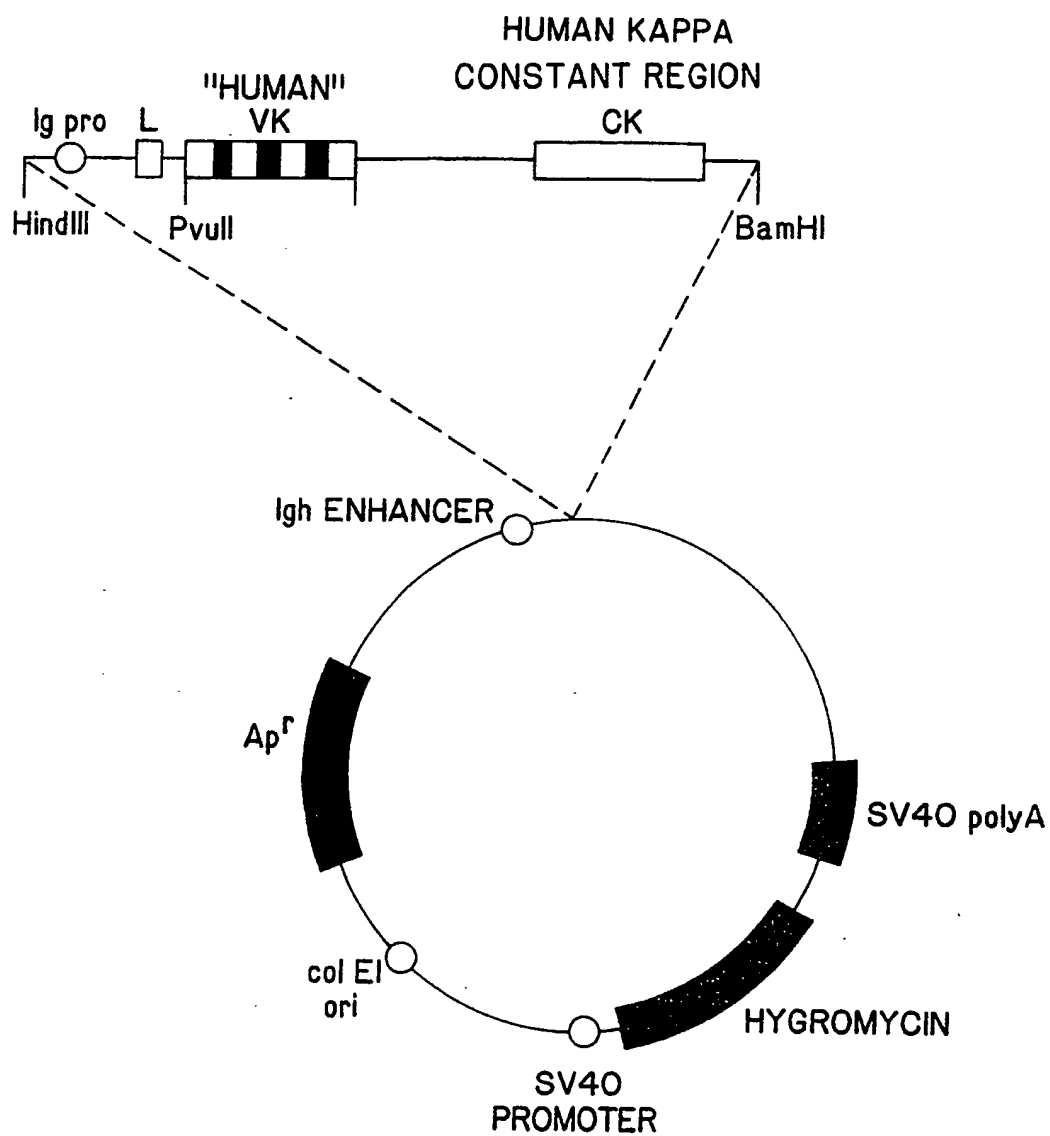


FIG. 4

RSV19VH	10	20	30	40	50	60
	QVQLQESGTE	LERSGAVKLS	CTASGFNIK	QVYMEIMK	QRPDQGLEWIG	MDPENDDVQYA-
	PKFQGGKAT	MTADTSSNT	AYLQLTSL	TFEDTAVYFC	NSMGSDFDH	MGQGTIVTVSS
	70	80	90	100	110	
pHuRSV19VH	QVQLQESGPGL	VRPSQTLSL	TCTVSGFTF	SDYYMEIM	VRQPPGRGLEWIG	MDPENDDVQYA-
	PKFQGGRTML	VDTSKNQFSL	RSLSSVTAAD	TAVYYCAR	MGSDFDH	MGQGTIVTVSS
	70	80	90	100	110	
pHuRSV19VHFNS	QVQLQESGPGL	VRPSQTLSL	TCTVSGFTF	SDYYMEIM	VRQPPGRGLEWIG	MDPENDDVQYA-
	PKFQGGRTML	VDTSKNQFSL	RSLSSVTAAD	TAVYFCNS	MGSDFDH	MGQGTIVTVSS
	70	80	90	100	110	
pHuRSV19VHNIK	QVQLQESGPGL	VRPSQTLSL	TCTVSGFNIK	QVYMEIM	VRQPPGRGLEWIG	MDPENDDVQYA-
	PKFQGGRTML	VDTSKNQFSL	RSLSSVTAAD	TAVYFCNS	MGSDFDH	MGQGTIVTVSS
	70	80	90	100	110	
RSV19VK	DIQLTQSP	LSPLVTLG	DQAISCRSS	QTLVHTDGN	TYLEMFLQ	KPGQSPKLLIYRVSNRFS-
	GVPDRFSG	SGSGTDF	TLKISRVEA	EDLGVYFC	QGGSHLPRIF	GGGKLEI
	10	20	30	40	50	
pHuRSV19VK	DIQLTQSP	SSLSASV	GDRVITITCR	SSQTLVHTDGN	TYLEMYYQ	KPKAPKLLIYRVSNRFS-
	GVPSRFSG	SGSGTDF	TFTISSLP	QEDTATYYC	QGGSHLPRIF	GGGKVEIK
	60	70	80	90	100	

FIG. 5

6 / 8

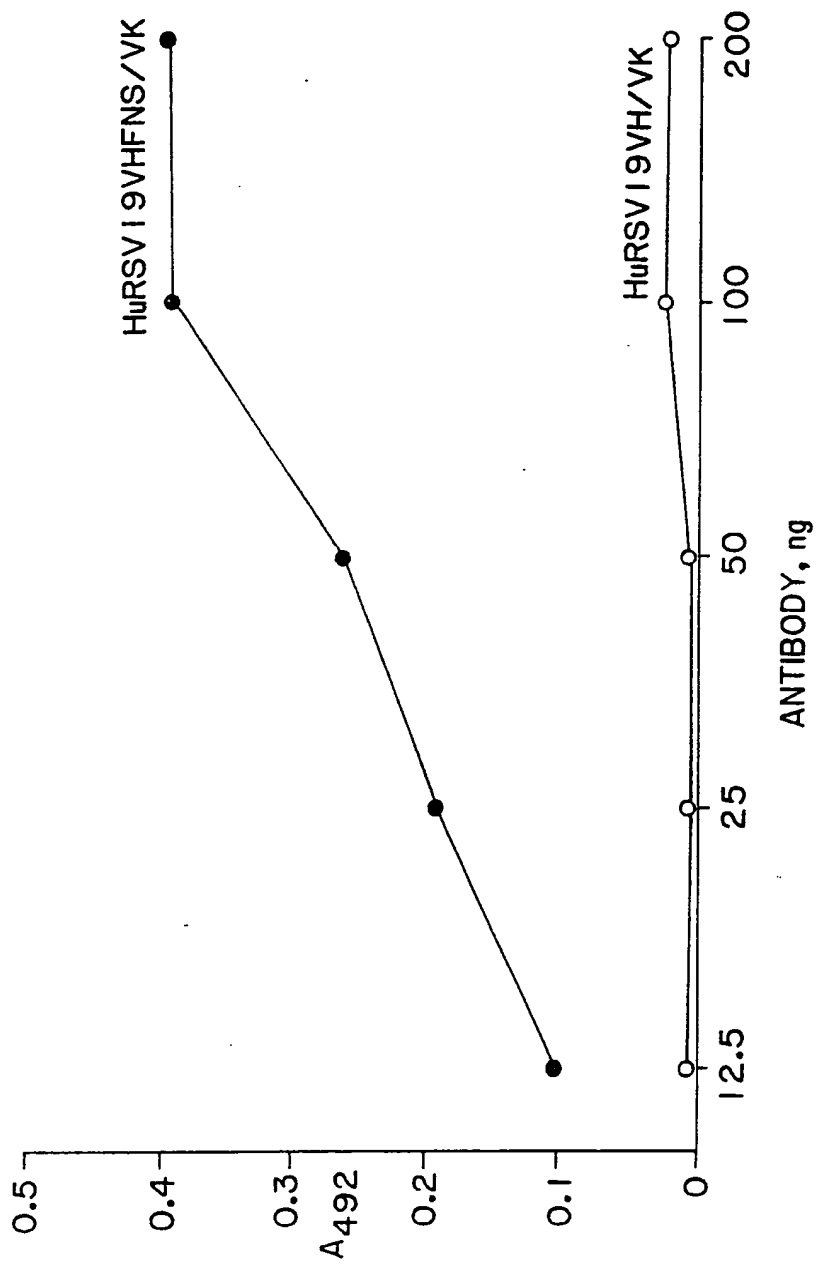


FIG. 6

7 / 8

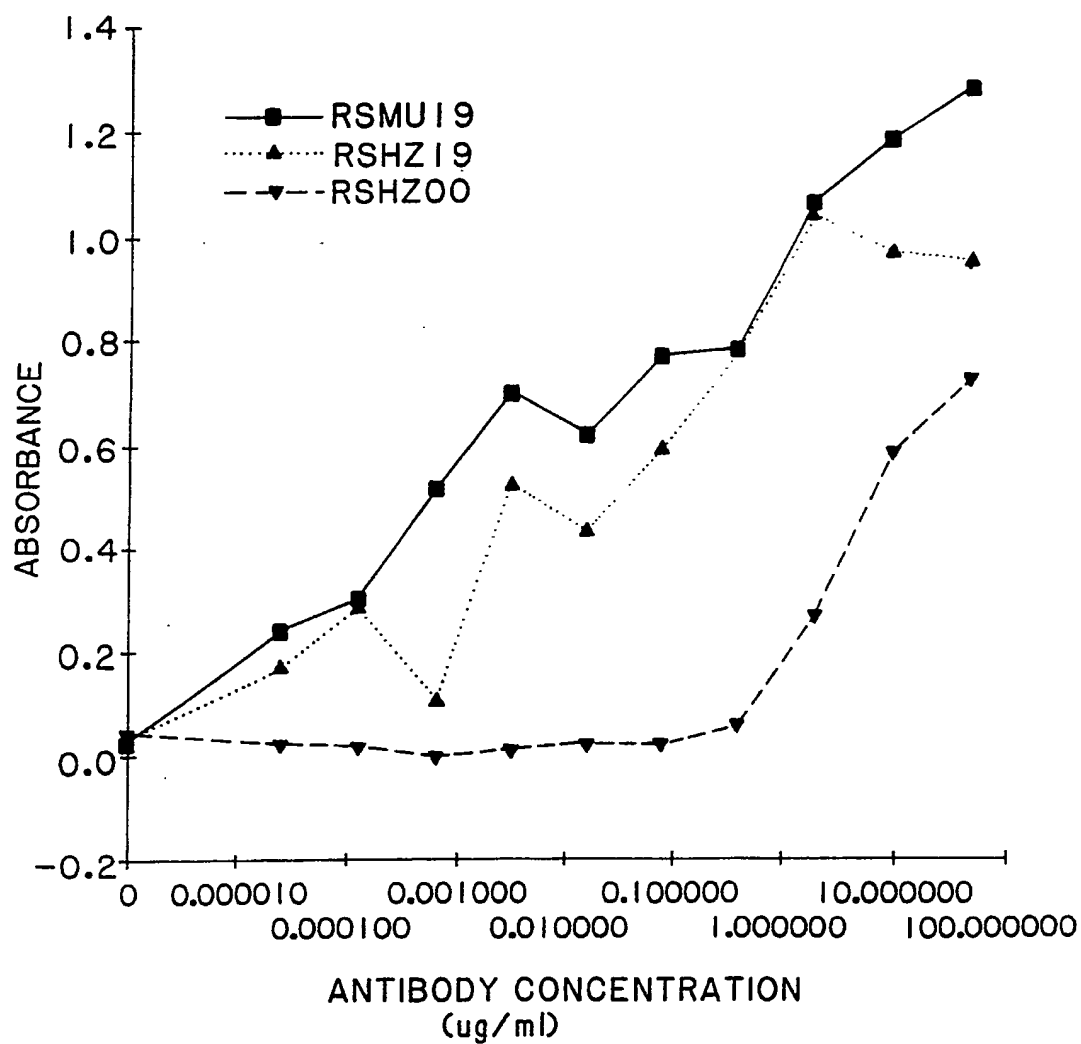


FIG. 6A

8 / 8

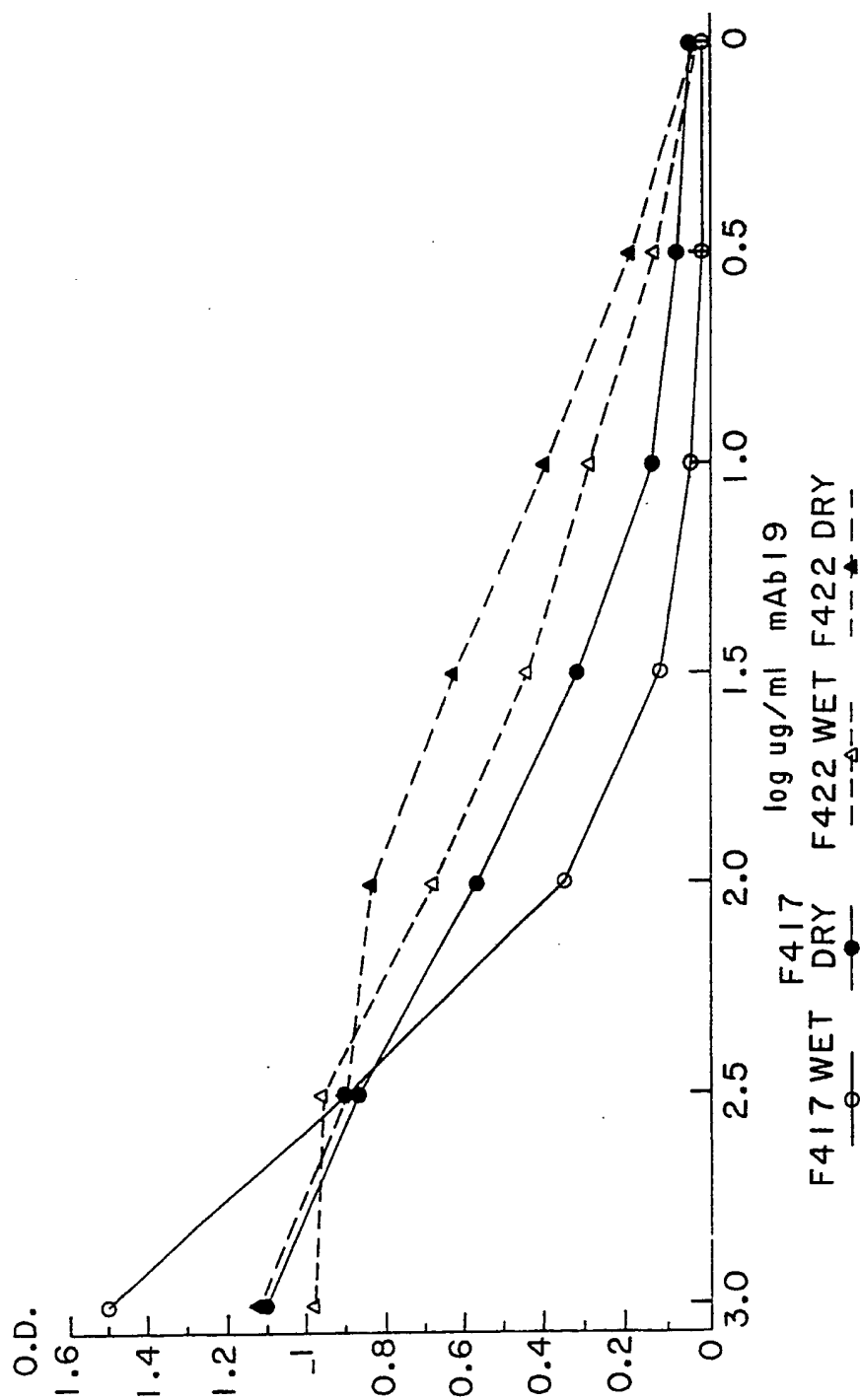


FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 91/01554

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl. 5 C 07 K 15/28 C 12 P 21/08 A 61 K 39/42
C 12 N 15/09 C 12 N 5/10

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
Int. Cl. 5	C 07 K C 12 P A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Proceedings of the National Academy of Sciences of USA, volume 86, no. 24, December 1989, Washington, DC, US, C. Queen et al.: "A humanized antibody that binds to the interleukin 2 receptor", pages 10029-10033, see abstract (cited in the application) ---	48
Y	---	1-21, 27 -29, 32- 34, 40, 43, 49- 52
Y	Canadian Journal of Microbiology, volume 32, no. 1, January 1986, (Ottawa, CA) M. Trudel et al.: "Immunovirological studies on human respiratory syncytial virus structural proteins", pages 15-21, see the whole article --- -/-	1-21, 27 -29, 32- 34, 40, 43, 49- 52

¹⁰ Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

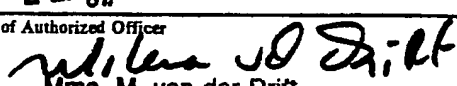
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09-12-1991	22.01.92
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer  Mma. M. van der Drift

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	US,A,4800078 (G. PRINCE et al.) 24 January 1989, see claim 1 ---	11-21, 27-29, 33,34, 40,49- 52
X,P	Biotechnology, volume 9, no. 5, May 1991 (New York, US) J. Hodgson et al.: "Making monoclonals in microbes", pages 421-425, see page 422, left-hand column, line 45 - middle column, line 54; page 423, right-hand column, lines 9-44 ---	1-21,27 -29,32- 34,40, 43,48- 52
A	Protein Engineering, volume 2, no. 3, September 1988 (Oxford, GB) J. Cheetham: "Reshaping the antibody combining site by CDR replacement-tailoring or tinkering to fit?", pages 170-172, see page 172, left-hand column, line 21 - right-hand column, line 19 -----	48

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim numbers 22-26,30,31,35-39,41,42, because they relate to subject matter not required to be searched by this Authority, namely: 44-47 Please see PCT Rule 39.1(iv)

*Remark: Although claims 21,27-29,34 and 40 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9101554

SA 51153

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 23/12/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4800078	24-01-89	None	